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Link(s) to article on publisher's website:
<http://dx.doi.org/doi:10.21954/ou.ro.0000f9d0>

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Cristiana Carniti

**STUDY OF THE RET RECEPTOR
DYSFUNCTIONS CAUSED BY MUTATIONS
ASSOCIATED WITH HUMAN NEOPLASTIC
DISORDERS AND DEVELOPMENTAL DISEASES**

Thesis submitted to the Open University of London for the
degree of Doctor of Philosophy

Discipline: Life Sciences

29 september 2003

ISTITUTO NAZIONALE TUMORI
MILANO-ITALY

DATE OF SUBMISSION 29 SEPTEMBER 2003
DATE OF AWARD 4 MARCH 2004

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ABBREVIATIONS

ARTN	Artemin
bFGF	basic fibroblast growth factor
bp	base pair
BSA	bovine serum albumin
CAD	cadherin
cDNA	complementary DNA
CNS	central nervous system
CYS	cysteine
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EDNRB	endothelin receptor B
ENC	enteric neural crest
ENS	enteric nervous system
ERK	extra-cellular signal regulated kinase
ES cells	embryonic stem cells
FMTC	familial medullary thyroid carcinoma
GDNF	glial cell line-derived neurotrophic factor
GFR α	GDNF family receptor alpha
GPI	glycosylphosphatidyl inositol
HSCR	Hirschsprung's disease
JNK	c-Jun N-terminal Kinase
Kb	kilobase pair
kDa	kilodalton
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MDCK	Madin-Darby canine kidney
MEN2	multiple endocrine neoplasia type 2
MTC	medullary thyroid carcinoma
mRNA	messenger ribonucleic acid
NC	neural crest
NRTN	neurturin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI3-K	phosphatidylinositol 3-kinase
PLC γ	phospholipase C γ
PNS	peripheral nervous system
PP1	pyrazolo-pyrimidine 1
PP2	pyrazolo-pyrimidine 2
PP3	pyrazolo-pyrimidine 3
PSPN	persephin

PTB	protein tyrosine-binding
PTC	papillary thyroid carcinoma
Ptyr	phosphor tyrosine
RET	rearranged during transfection
RTK	receptor tyrosine kinase
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH2	Src homology-2
TBS	tris buffered saline
TK	tyrosine tinase
TM	trans-membrane
Tyr	tyrosine
UB	ureteric bud
WCL	whole cell lysates

ABSTRACT

The *ret* proto-oncogene encodes a membrane spanning glycoprotein which is a member of the receptor tyrosine kinase family (Hanks et al. 1988). RET is the signaling component of multi-subunit receptor complexes for the GDNF of family ligands, including GDNF, neurturin, artemin and persephin. The binding components of these receptor complexes are glycosyl-phosphatidylinositol (GPI)-membrane anchored molecules, known as GDNF family receptor α (GFR α s). Four different GFR α s (GFR α 1-4) dictate ligand specificity.

Germline point mutations of RET are responsible for the inheritance of MEN2 (Multiple Endocrine Neoplasia type 2) cancer syndromes which are usually divided into three different clinical subtypes: MEN2A, MEN2B and FMTC (familial medullary thyroid carcinoma), which are all autosomal dominant cancer syndromes. Inactivating mutations of RET cause an impaired development of the enteric nervous system which is responsible for the Congenital megacolon or Hirschprung's disease (HSCR).

The aim of my work was to study the expression of different RET mutants in order to highlight their biological role in diverse cellular context. In particular, we focused on gain of function cysteine mutations that are responsible for medullary thyroid carcinoma (MTC) by causing covalent RET dimerisation, leading to ligand-independent activation of its tyrosine kinase. In this context, the association of

Cys⁶⁰⁹ and Cys⁶²⁰ activating mutations with HSCR is still an unresolved paradox. To address this issue, we have developed a transgenic model for human diseases (specifically, Multiple Endocrine Neoplasia type 2 and Hirschsprung disease) through the insertion of a gain and loss of function RET mutation, the RET^{C620R} in the mouse genome. We have also studied the *in vitro* effects of a tyrosine kinase inhibitor PP1, which we propose could represent a potential treatment strategy and merits further testing, using *in vivo* models such as the one we have generated.

1. REVIEW OF THE LITERATURE

1.1 RET PROTO-ONCOGENE AND ITS PHYSIOLOGICAL FUNCTIONS

1.1.1 Identification of RET as an oncogene

The ret proto-oncogene encodes a membrane spanning glycoprotein which is a member of the receptor tyrosine kinase family (Hanks et al. 1988). It was first identified as a component of a chimeric oncogene when DNA from lymphomas, colon and gastric tumours produced transformation of NIH3T3 cells (Takahashi et al, 1985). These rearrangements were an artefact of the transfection assay. The chimeric protein comprised an N-terminal region with a motif for dimerization fused to a new tyrosine kinase domain that afterwards was found to belong to a transmembrane receptor named RET (REarranged during Transfection) (Takahashi et al, 1988).

The RET gene lies on chromosome band 10q11.2 and comprises 21 exons spanning 55kb. The gene encodes a protein similar to other TK receptors (Ishizaka et al, 1989). Homologues of RET have been identified in higher and lower vertebrates, as well as in *Drosophila Melanogaster* (Hahn and Bishop, 2001).

1.1.2 Structure and function of the RET receptor tyrosine kinase

RET protein is characterized by an N-terminal signal peptide, a cadherin-like motif and a cysteine rich region in the extracellular domain, a transmembrane domain and the intracellular domain that contains the tyrosine kinase domain interrupted by 27 amino acid of the kinase insert (Itoh et al, 1992; Schneider, 1992). Its structure is similar to the ones of other tyrosine kinase receptors, apart from the presence of the cadherin domain (Schneider, 1992). Cadherins are Ca^{2+} -dependent cell-cell adhesion proteins and their adhesive property depends on a domain of 110 amino acids, repeated in tandem, in the extracellular region. The binding of calcium between each cadherin domain is believed to induce linearization and strengthening of the whole extracellular region, thus protecting cadherins from proteolytic degradation. Multiple sequence alignments and computer modelling analyses have revealed that the extracellular domain of RET comprises four repeated cadherin-like domains (Anders et al, 2001). In addition, it has been demonstrated that the extracytoplasmic domain of RET binds specifically to Ca^{2+} ions, thus confirm the association of RET with the cadherin super family. Remarkably RET does not fold correctly inside the endoplasmic reticulum when extracellular calcium is depleted (van Weering et al, 1998), and binding of ligands is calcium-dependent (Nozaki et al, 1998). Together, these data suggest that the

fixation of calcium through the cadherin domains of RET induces, or stabilizes, a conformational change in the extracytoplasmic domain, which is necessary for the interaction with its cognate ligands.

RET is expressed as either a 150kDa or a 170kDa protein, respectively corresponding to the immature incompletely glycosylated form present in the endoplasmic reticulum and to the mature RET protein, fully glycosylated and expressed at the cell surface (Taniguchi et al, 1991). The C-terminal tail, starting from aa 1062, shows three different splicing variants: the long isoform of 1114 aa (Iso51), the middle isoform of 1106 aa (iso 43) and the short isoform of 1072 aa (iso9) (Tahira et al, 1990; Myers et al, 1995).

In situ hybridization showed that RET is present in peripheral enteric, sympathetic and sensory neurons. RET staining is also observed in central motor, dopamine and noradrenalin neurons localized in the ventral half of the spinal cord, in the neuroretina and in the olfactory epithelium (Pachnis et al, 1993; Attie-Bitach et al, 1998; Manie et al, 2001). In these locations RET activation can promote neuronal cell survival and differentiation. Outside the nervous system, RET is involved in renal ontogenesis, in particular in mesonephric duct and branching ureteric bud development, where a chemotactic role for GDNF has been demonstrated (Taraviras and Pachnis, 1999) although RET transcripts disappear after birth. All these observations are

supported by studies on RET- and coreceptor-null mice, or on transgenic mice with a defective RET TK domain that show severe defects of the innervation of the hindgut and branching of the ureteric bud (Airaksinen et al, 1999; Baloh et al, 2000).

1.1.3 Different splice isoforms of RET

In mammals, c-RET is alternatively spliced to produce at least two major isoforms, RET9 and RET51, that differ only in the amino acid sequence of the C-terminal tail. The two RET isoforms are highly conserved between species, suggesting that these regions have important functions that are conserved in evolution. Although the two isoforms behave similarly in a number of in vitro assays, several observations have suggested that they have different and tissue-specific effects on embryogenesis and tumorigenesis. Monoisoformic mouse strains expressing only RET9 or RET51 in place of the normal complement of c-Ret gene products, demonstrated that signaling by RET9 is critically important for kidney morphogenesis and enteric nervous system development and postnatal life: signaling by RET51 alone, in the absence of RET9 resulted in characteristic defects in the development of the excretory and enteric nervous systems (de Graaff et al, 2001) indicating that RET51 is dispensable during embryogenesis. Furthermore, transgenic overexpression of RET51 only

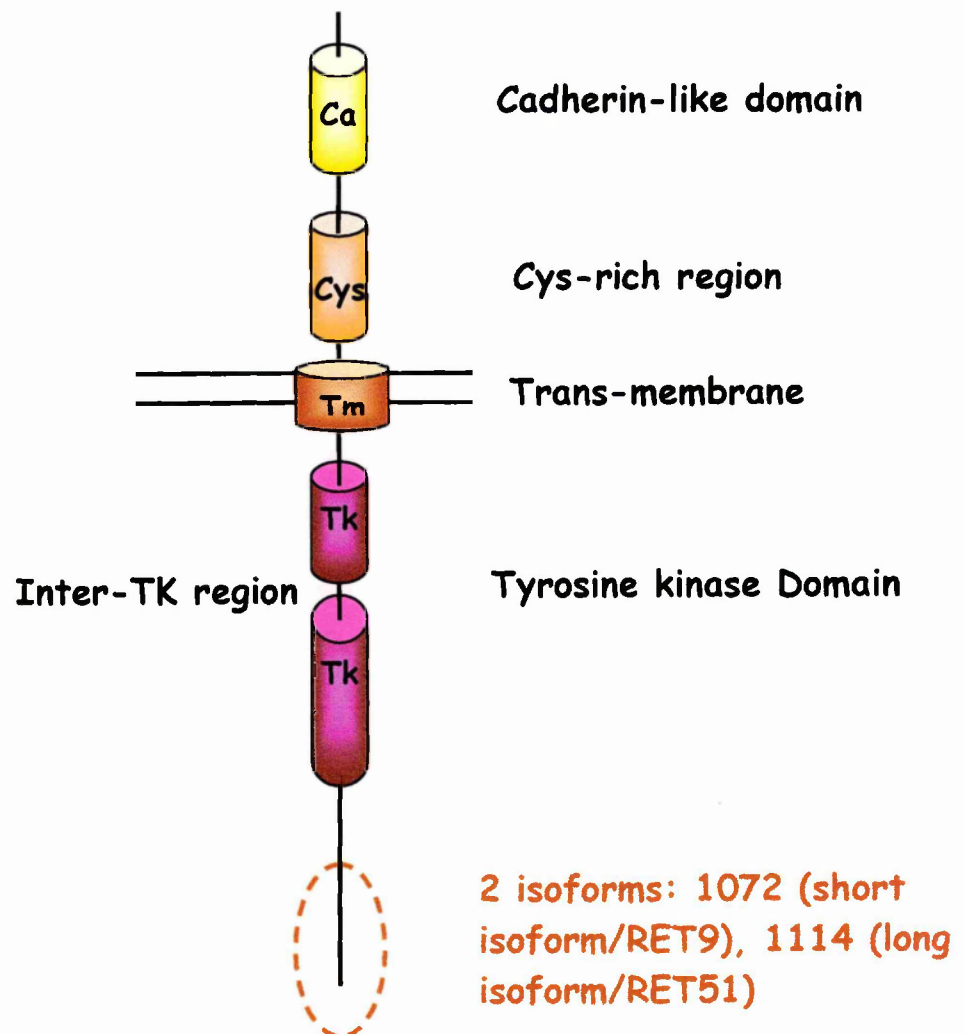


Figure1. RET structure

partially compensates for the loss of RET9 in kidney and enteric nervous system development. RET51, but not RET9, is required for the metabolism and growth of mature sympathetic neurons (Tsui-Pierchala et al, 2002b). Moreover, Tsui-Pierchala et al (Tsui-Pierchala et al, 2002a) provided evidence that RET9 and RET51 are not only functionally distinct, but also activate a distinct assortment of signaling pathways in neurons and are not able to associate with each other after GDNF stimulation probably due to different subcellular localization of the two isoforms. The alternative splicing of RET might account for an evolutionarily conserved mechanism to expand the number of activities regulated by a RTK.

1.1.4 Identification of ligands and co-receptors for RET

The RET ligand was unknown until 1996 when the Glial Derived Neurotrophic Factor (GDNF) was discovered (Trupp et al, 1996; Durbec et al, 1996). The first member of the GDNF family was identified due to the ability of conditioned media, from glial cell line culture, to promote the survival of dopaminergic neurones. Mice null for GDNF were found to display a very similar phenotype to Ret-null mice, in terms of renal agenesis, colon aganglionosis and absence of superior cervical ganglia. GDNF was also shown to cause RET phosphorylation and exert trophic effects on RET-expressing tissue explants (Sanchez et al, 1996; Pichel et al, 1996; Moore et al, 1996; Trupp et al, 1996). Nevertheless, no direct interaction of RET and GDNF could be demonstrated and a co-receptor was subsequently identified as being necessary for GDNF mediated activation of RET (Jing et al, 1996; Sanicola et al, 1997). GDNF was the first of a family of 4 ligands (to date) comprising Neurturin (NTN) (Kotzbauer et al, 1996), Persephin (PSP) (Milbrandt et al, 1998) and Artemin (Baloh et al, 1998b; Baloh et al, 1997; Sanicola et al, 1997; Jing et al, 1997). They represent a new subclass of the transforming growth factor TGF β superfamily and like members of this family, glial-cell-line-derived neurotrophic factor ligands are secreted as disulphide-linked dimers

that contain three disulphide bonds arranged in a typical configuration known as "cysteine knot" (Eigenbrot et al, 1997). All these factors are structurally closely related, sharing 40% identity at the amino acid level. Similar to neurotrophins, the GDNF family of ligands are also firstly synthesized as precursor polypeptides that are processed to mature proteins and then secreted (Lin et al, 1993).

GDNF was described originally as a trophic factor for dopaminergic neurons (Lin et al, 1993), although now it seems to be involved in the survival of a wide spectrum of neurons, including motor, noradrenergic, enteric, parasympathetic, sympathetic and sensory neurons (Airaksinen et al, 1999; Baloh et al, 2000). NTN, ARTN and PSPn share similar neurotrophic effects to GDNFs although PSPN does not support the survival of peripheral neurons (Airaksinen et al, 1999; Baloh et al, 2000).

RET signal starts from a multimeric complex composed of the RET kinase and any one of four different high-affinity glycosyl-phosphatidylinositol (GPI)-linked coreceptors, designated as GFR α 1,2,3 and 4 (Jing et al, 1996; Treanor et al, 1996; Klein et al, 1997; Buj-Bello et al, 1997; Baloh et al, 1997; Jing et al, 1997; Baloh et al, 1998a; Enokido et al, 1998; Thompson et al, 1998). They define a structurally related subfamily, although alternatively spliced forms of GFR α -4 have been predicted to encode transmembrane and soluble

isoforms (Lindahl et al, 2000; Masure et al, 2000). The interactions between the identified ligands and coreceptor proteins have been assayed using different methods such as the equilibrium binding of radiolabeled ligands to coreceptors, the ability to promote survival of neurons microinjected with each of the coreceptors and the ability to induce RET autophosphorylation. It has been demonstrated that the four RET ligands GDNF, NTN, PSP and Artemin interact preferentially with GFR α 1, 2, 3 and 4 respectively (Creedon et al, 1997; Baloh et al, 1997; Sanicola et al, 1997; Jing et al, 1997). Nevertheless, equilibrium binding data suggest that a low affinity interaction was also possible and that there was a degree of cross-talk between ligands and coreceptors (Baloh et al 1997, Jing et al 1997, reviewed by Saarma 2000), except that mammalian GFR α -4 coreceptor binds only PSPN (Lindahl et al, 2001).

A. Glial Derived Neurotrophic Factor (GDNF)

Transcription of the gene usually produces a full-length GDNF mRNA of 4.5kb, but a 6.0 kb transcript mRNA has also been observed and encodes a 211 amino acid precursor polypeptide, from which the mature protein of 134 amino acids is produced by proteolytic cleavage (Lin et al, 1993). There are two potential glycosylation sites in the mature protein, which has a Mr of 18 to 22 kDa (Lin et al, 1993).

GDNF promotes the survival of several types of neurons in both the central and peripheral nervous systems. Exogenous GDNF can maintain dopaminergic, noradrenergic and motor neurones of the central nervous system (Lin et al, 1993; Henderson et al, 1994; Arenas et al, 1995; Messer et al, 2000; Rosenblad et al, 2000), as well as various sub-populations of peripheral sensory and sympathetic neurones (Henderson et al, 1994; Buj-Bello et al, 1995; Ebendal et al, 1995; Trupp et al, 1995; Arce et al, 1998). The pharmacological effects of GDNF on midbrain dopaminergic neurones have been studied and GDNF is considered a potential drug candidate for the treatment of Parkinson's disease. (reviewed by Lapchak et al, 1997; Grondin et al, 1998).

Transgenic models have demonstrated that the development of dopaminergic neurones in GDNF-deficient embryos is normal. However, homozygous mice die at birth before the maturation of dopaminergic nervous system. GDNF-deficient mice show a 20%-30% reduction in the number of motor neurones in the trigeminal ganglia and the spinal cord (Moore et al, 1996; Sanchez et al, 1996). Transgenic mice over expressing GDNF under a muscle-specific myogenic promoter show hyper innervations of neuromuscular junctions for several weeks after birth (Nguyen et al, 1998),

suggesting that GDNF also functions as a muscle-derived factor for motor axon branching and synapse elimination.

Outside the nervous system, GDNF mRNA is abundantly expressed in condensing nephrogenic mesenchyme cells around the tips of the invading ureteric buds, while branching bud tips express the functional receptor RET (Pachnis et al, 1993; Widenfalk et al, 1997). The specific expression pattern of GDNF and RET suggested a crucial paracrine regulatory role of GDNF in kidney morphogenesis. GDNF-deficient mice lack kidneys and die in the first postnatal day (Moore et al, 1996; Pichel et al, 1996).

GDNF increases motility, dissociation of cell adhesion and migration of the RET-transfected Madin-Darby canine kidney (MDCK) cell line toward a localised source of GDNF (Tang et al, 1998) and promotes scattering of the RET-transfected human neuroectodermic SK-N-MC cell line.

B. Neurturin (NRTN)

NTRN, with 42% similarity to GDNF at the amino acid level, was identified on the basis of its ability to support the survival of sympathetic postganglionic neurones in culture (Kotzbauer et al, 1996). NRTN promotes the survival of several populations of neurones in both the central and peripheral nervous systems (Klein et al, 1997).

There is a difference between the effect of GDNF and NRTN on developing and adult substantia nigra dopaminergic neurones; GDNF has wider survival, neuritogenic and hypertrophic effects when compared to the selective survival-promoting effects of NRTN (Akerud et al, 1999; Rosenblad et al, 1999a,b). NRTN affects parasympathetic neuronal survival and targets innervations. NRTN-deficient mice have a strikingly similar phenotype to mice lacking $GFR\alpha 2$, showing defects in the parasympathetic enteric nervous system (Rossi et al, 1999). They are viable and fertile, but have a dramatically reduced myenteric plexus innervation. NRTN and $GFR\alpha 2$ together with RET, regulate the parasympathetic innervation of the penis and may also have a role in the regulation of heart innervation (Laurikainen et al, 2000, Hiltunen et al, 2000).

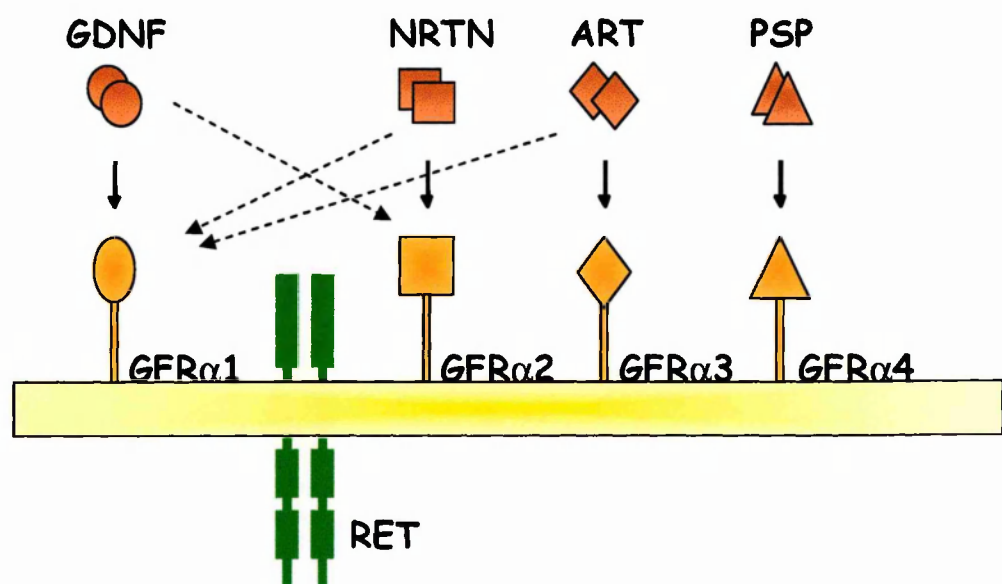


Figure 2. The binding of GDNF family ligands and receptors. RET is the signaling component of multisubunit receptor complexes for the GDNF family ligands that includes GDNF, neurturin, artemin and persephin. The binding components of these receptor complexes are glycosyl-phosphatidylinositol (GPI)-membrane anchored molecules, known as GDNF family receptor α (GFR α s). Four different GFR α s (GFR α 1-4) dictate the ligand specificity of the complex. The solid arrows represent the functional binding that activates RET most potently, whereas the dotted arrows indicate weak interactions with receptor.

1.1.5 Recruitment of RET to lipid rafts and action of GDNF

Lipid rafts are sphingolipid- and cholesterol-rich membrane domains thought to act as scaffolding centres which mediate the assembly of a specific set of signal transducers (Tansey et al, 2000). In fact, raft micro domains might help to compartmentalise sets of signaling molecule at both sides of the plasma membrane, allowing them to interact with each other in a regulated manner, and at the same time preventing them from interacting with proteins excluded from rafts (Simons and Toomre 2000). It was demonstrated that inactive RET is localised outside rafts (Paratcha et al, 2001, reviewed

by Saarma 2001). Glycosyl phosphatidylinositol-anchored molecules are known to be located within lipid rafts and, consistent with this, GFR α -1 recruits RET to lipid rafts upon treatment with GDNF (Tansey et al, 2000). Moreover, depletion of cholesterol with methyl- β -cyclodextrin causes disruption of the raft structure and decreases GDNF signalling, indicating that compartmentalization of RET and GFR α -1 in lipid rafts is crucial for transduction of the GDNF signal. It is therefore possible that even the other GFR α proteins are able to recruit RET into lipid rafts.

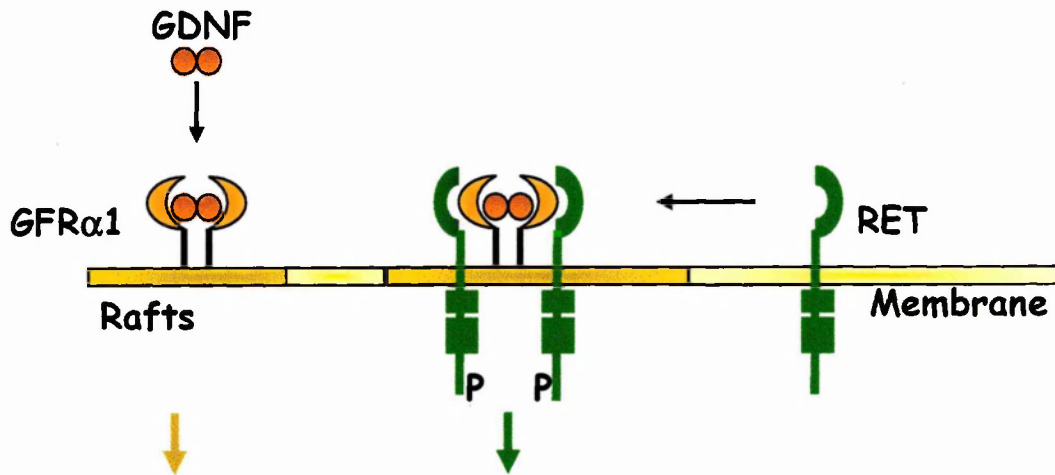
1.1.6 RET activation

Current experimental data favour the existence of two models for the activation of RET, including *in cis* signalling and *in trans* signalling mechanisms. Originally the proposed model for RET activation states that the ligand-receptor complex is assembled in a step-wise fashion (Jing et al, 1996). A dimeric GDNF molecule induces or stabilizes the formation of a complex with two monomers of GFR α 1. In a second step, this complex contacts two molecules of RET and promotes their homodimerization and autophosphorylation at tyrosine residues (in *cis* signalling) (Paratcha et al 2001 and reviewed by

Saarma 2001). GFR α 1 recruits RET to lipid rafts only following GDNF stimulation, resulting in the association of RET and Src. The overlapping and complementary expression of GFR α s with each other, with ligands and with RET, suggests that the functions of the GDNF family can be regulated in various ways. GFR α 1 is widely expressed in the absence of RET, suggesting alternative roles for "ectopic" sites of GFR α 1 expression. GFR α 1 also binds its ligand and activates RET when provided exogenously in soluble form or immobilised on agarose beads (Treanor et al, 1996; Yu et al, 1998; Qiao et al, 1999). GFR α s are usually bound to the plasma membrane but a specific cleavage by a putative phospholipase or protease produces soluble forms of these co-receptors (Paratcha et al, 2001). Thus, another possibility is that GFR α 1 may also function in a non-cell-autonomous manner to capture and concentrate diffusible GDNF family ligands from the extracellular space and then present these factors *in trans* to affect RET-expressing cells (*in trans* signalling) (Paratcha et al, 2001; Saarma et al, 2001). Activation of RET *in trans* also results in the mobilisation of RET to lipid raft membranes even in cells that lack endogenous GPI-anchored GFR α 1 (Paratcha et al, 2001). Upon activation *in cis*, RET is recruited to membrane rafts by a rapid extracellular mechanism, most likely driven by its affinity for newly formed GDNF/GFR α 1 complexes, independently of its tyrosine kinase activity. In contrast, recruitment *in*

trans is delayed, sustained, and requires an active RET tyrosine kinase, suggesting the involvement of intracellular events (Paratcha et al, 2001). Downstream signalling after stimulation *in trans* is at least as efficient as that induced *in cis* and potentiates downstream signalling, neuronal survival and differentiation (Paratcha et al, 2001). Based on the existing data it is possible to assume that also other members of the GDNF family, Neurturin, Artemin and Persephin interact with their cognate co-receptor and activate RET similarly to GDNF.

a)



b)

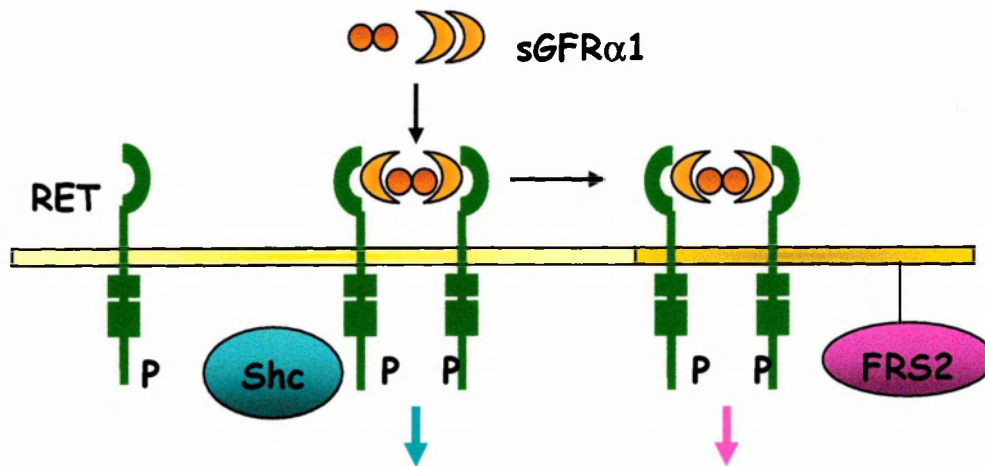


Figure 3. Possible mechanisms of RET activation. a) *cis* signaling: GFR α 1 recruits RET to lipid rafts, after treatment with GDNF (tyrosine kinase independent process). b) *trans* signaling: GDNF binds to soluble GFR α 1 and the GDNF- GFR α 1 complex triggers RET activation Trans

activated RET is located both outside and in the lipid compartment (tyrosine kinase dependent process).

Intracellular signaling through RET has been extensively studied (reviewed by Airaksinen et al, 1999; Takahashi 2001). Since RET ligands were unknown until 1996, extensive studies on RET signalling have been performed using chimeric or oncogenic version of the transmembrane RET, that display constitutive tyrosine kinase activity. In fact the activation of the signalling cascade starts upon ligand binding that induces receptor autophosphorylation and consequent the phosphorylation of tyrosine residues. RET can activate various signaling pathways including RAS/extra cellular signal-regulated kinase (ERK), phosphatidylinositol 3 kinase (PI3K)/AKT, p38 mitogen activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways (Airaksinen et al, 1999; Trupp et al, 1999; Hayashi et al, 2000). These signals are transmitted through phosphotyrosines present in the intracellular domains of RET that mediate specific binding to various scaffolding, anchoring, adaptor proteins and enzymes that possess Src homology-2 (SH2) or protein tyrosine-binding (PTB) domains. The RET C-terminal tail comprises five different phospho-tyrosine residues (Y687, Y826, Y1015, Y1029 and

Y1062) apart from the TK. In addition, the long isoform displays two extra tyrosine residues: Y1090 and Y1096; the latter is phosphorylated in RET/2A but not in RET/2B (Liu et al, 1996). In particular, Y1062 is a multidocking site interacting with a number of transduction molecules: SHC, FRS2, IRS1/2, DOK proteins (Arighi et al, 1997; Kurokawa et al, 2001) and Enigma (Durick et al, 1998) and has a critical role in signaling initiation during embryogenesis and tumorigenesis (reviewed by Hayashi et al, 2000; Takahashi 2001). All the RAS/ERK, PI3K/AKT, p38MAPK and JNK pathways are activated mainly through tyrosine 1062 that is the binding site for SHC adaptor proteins. After binding of SHC to tyrosine 1062, SHC further associates with GAB1/2 adaptor proteins and the GRB2/SOS complex, leading to the activation of PI3K/AKT and RAS/ERK signaling pathways, respectively. SNT/FRS2 is a lipid anchored docking protein with a phosphotyrosine-binding (PTB) domain, and it may be involved mainly in activation of the RAS/ERK signaling pathway. The p38MAPK, JNK pathways are also activated through tyrosine 1062. Moreover, RET signals also through PLC γ , that binds Y1015 (Borrello et al, 1996) and through GRB2 that binds only the long isoform at Y1096 (Alberti et al, 1998)

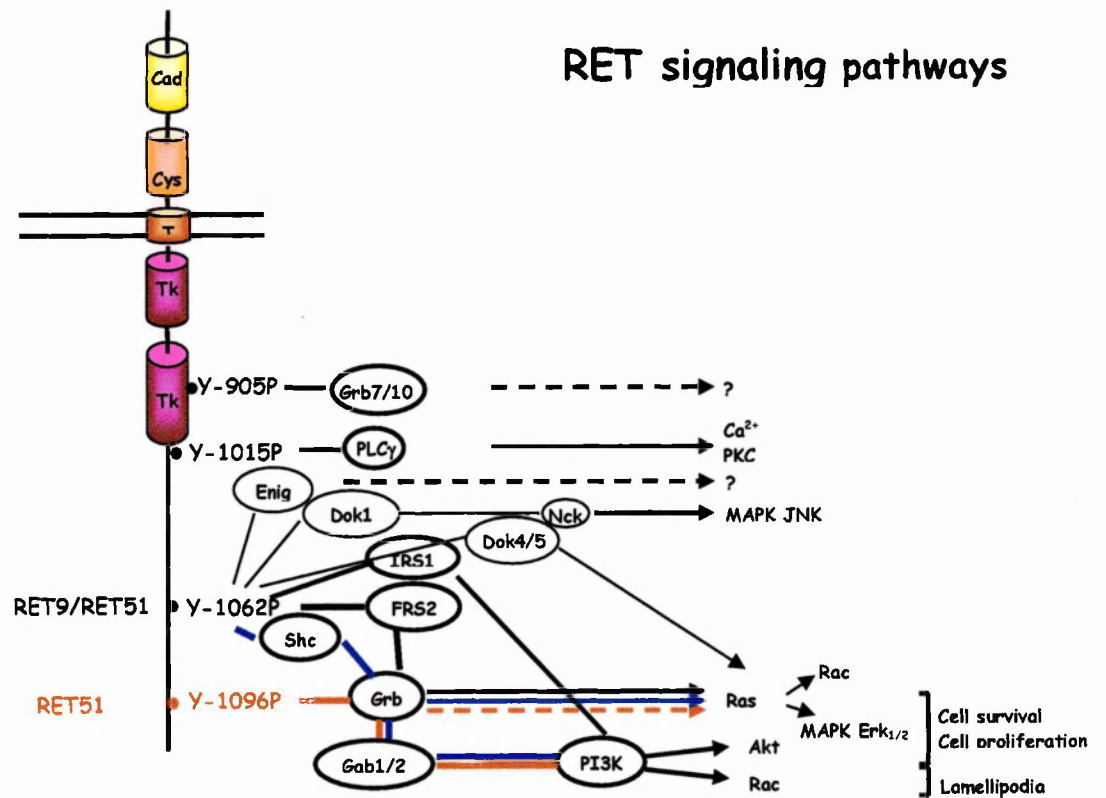


Figure 4. RET signaling pathways. Activation of RET leads to autophosphorylation of tyrosine residues (Y-P) within the cytoplasmic tail, creating docking sites for multiples signaling partners.

1.2 RET IN HUMAN PATHOLOGIES

1.2.1 RET oncogenes in papillary thyroid cancer

Chromosomal rearrangements producing chimeric oncogenes are frequently associated with human cancer. Several lines of evidence suggest that they are involved in the pathogenesis of tumors. Among solid tumors, papillary thyroid carcinoma (PTC) provides a unique model of a frequent generation of chimeric oncogenes through chromosomal rearrangements. Transfection of DNA from papillary thyroid carcinomas into NIH3T3 cells led to the identification of rearrangements of RET tyrosine kinase receptor with foreign sequences. In fact, PTCs are characterized by the generation of fusion proteins, made of the N-terminus derived from different partners and the C-terminus of one of the two receptors, carrying a cytoplasmic tyrosine kinase domain.

In 1987, Dr. Pierotti's group in Milan, in collaboration with the group headed by G Vecchio and A Fusco of the University of Naples, isolated the first activated version of the RET oncogene, named RET/PTC (Papillary Thyroid Cancer) (Fusco et al, 1987). Afterwards, a number of different RET/PTCs have been isolated, where RET TK domain was found fused with different partner genes.

A. RET/PTC1

RET/PTC1 originates from an inversion of chromosome 10, inv(10)(q11.2q21.2), and results from the fusion of the RET-TK domain and H4 (D10S170) gene, whose function is still unknown. The H4/RET fusion incorporates 101 amino acids of H4, predicted to encode a leucine zipper domain responsible for RET/PTC1 oligomerization and constitutive tyrosine kinase activity (Tong et al, 1997). A novel rearrangement containing the N-terminal 150 residues of H4 creates an oncoprotein named RET/PTC1L able to transform NIH3T3 cells with 5-fold lower efficiency than RET/PTC1. Its low transforming ability may be one of the reasons explaining its low frequency in human thyroid carcinomas (Giannini et al, 2000). Recently H4 has been found fused to the platelet-derived growth factor receptor beta gene in atypical chronic myeloid leukemia (t(5;10)(q33;q22) (Schwaller et al, 2001). Interestingly RET/PTC1 rearrangement has been found associated with post-Chernobyl PTC of long latency.

B. RET/PTC2

In RET/PTC2, RET-TK is fused to the type I alpha regulatory subunit of protein kinase A (RI alpha) and is generated by a reciprocal

and balanced chromosome translocation (Lanzi et al, 1992; Bongarzone et al, 1993). The resulting 596-aa protein contains the first two-thirds of RI α . The wild-type RI α subunit dimerizes in an antiparallel orientation between Cys-16 and Cys-37 (Bubis et al, 1987). RET/PTC2 deletion mutants showed that the RI α dimerization domain is the only portion of RI α required for RET/PTC2 mitogenic activity, thus suggesting that RET TK is activated in RET/PTC2 via the dimerization domain of RI α (Durick et al, 1995).

C. RET/PTC3

Both RET/PTC3 and RET/PTC4 oncogenes are generated by an intrachromosomal rearrangement with the ELE1 α /ARA70 gene. RET/PTC3 contains the first 238 amino acids of the androgen receptor-associated protein 70 (Santoro et al, 1994). Bongarzone et al. (Bongarzone et al, 1997) identified a short homology sequence (3 to 7 bp) in the two rearranging genes and a break cluster region (bcr) in ELE1, in A+T rich regions (Bongarzone et al, 1997).

The N-terminal coiled-coil domain of ELE1 α /ARA70 mediates oligomerization, RET kinase activation and transforming ability. In fact, expression of RET/PTC3 mutants lacking the N-terminal coiled-coil domain does not lead to foci formation in NIH3T3 cells. Moreover, the same domain mediates the interaction between RET/PTC3 and ELE1 α

/ARA70, causing oncoprotein re-localization to the plasma membrane (Monaco et al, 2001). Intrachromosomal rearrangements involving RET and the adjacent H4 or ELE1 α /ARA70 gene on chromosome 10 are very frequent events in thyroid cancer of children from the Chernobyl-contaminated zone (58%) (Klugbauer et al, 1995; Nikiforov et al, 1997). In addition, RET/PTC3 rearrangement is strongly associated with PTC of short latency and connected with a solid-follicular variant of PTC (Thomas et al, 1999). Consistent with this, mice carrying RET/PTC3 display an aggressive tumor phenotype, including competence for lymph node metastases (Powell et al, 1998). In contrast, RET/PTC1 transgenic mice develop follicular hyperplasia and carcinoma, but not invasive cancer (Santoro et al, 1996). In RET/PTC3 transgenic mice thyroids, a high frequency of solid-type papillary carcinomas was found, similarly to what was observed in thyroids of PTC3 positive patients from the Chernobyl-contaminated area. This suggests that the RET/PTC3 gene is critical for the development of the solid subtype of papillary thyroid cancer. Recently, Basolo et al associated the RET/PTC3 rearrangement also with the Tall-Cell Variant of papillary thyroid carcinoma. The finding that RET/PTC3 is present in aggressive histological PTC subtypes (Solid PTC and TCV) could depend on a more efficient *in vitro* mitogenic ability and MAPK

activation of this RET rearrangement in comparison to RET/PTC1 (Basolo et al, 2002).

D. RET/PTC4

In the case of RET/PTC4, despite the presence of the same RET/PTC3 breakpoint in exon 5 of the ELE1 α /ARA70 gene, the sequence of the rearranged genomic DNA showed a different intra-exonic breakpoint in the RET proto-oncogene. Moreover, it has been demonstrated that exon 5 of ELE1 α /ARA70 is joined to exon 11 instead of exon 12 of the RET gene. As a consequence, the RET/PTC4 cDNA sequence is 93 nucleotides longer than that of RET/PTC3 (Fugazzola et al, 1996).

E. Other PTCs

After the Chernobyl power plant explosion, an unusual number of thyroid cancers was noted in Belarus and Ukraine, between 10 to 30 fold higher than in the rest of Europe. The analysis of PTCs derived from the contaminated zones led to the identification of other rearranged forms of RET, where RET-TK was fused to 7 different donor genes. For instance, the RET/PTC5 fusion partner protein is GOLGA5, a coiled-coil protein expressed on the Golgi surface (Klugbauer et al, 1998). RET/PTC6 and RET/PTC7 respectively display rearrangements

with the Transcriptional Intermediary Factor 1-Alpha and Gamma (Klugbauer and Rabes, 1999). This protein family is able to bind to the ligand-dependent activation function (AF2)-activating domain of the estrogen receptor, RARs, RXRs, the vitamin D3 receptor and regulate transcription. Kinectin is the RET/PTC8 partner (Salassidis et al, 2000), whereas Rfg9, a putative cytoplasmic protein that might be involved in intracellular transport processes, is rearranged with RET to form RET/PTC9 (Klugbauer et al, 2000). In RET/PCM-1 the activating sequences belong to a gene coding for a centrosomal protein that displays distinct cell cycle distribution (Corvi et al, 2000). The last RET rearrangement found is ELKS/RET. ELKS mRNA is ubiquitously expressed, with the highest expression in heart, placenta, pancreas, thyroid and testis, but the function of the ELKS protein is still unknown (Nakata et al, 1999).

ONCOGENE	ACTIVATING GENE	ACTIVATING GENE FUNCTION	CHROMOSOME
PTC1*	H4/D10S170	Unknown	10q21
PTC2	RI α (PKA)	PKA regulatory subunit	17q23-q24
PTC3*- PTC4*	ELE1	Androgen receptor-associated protein (co-transcription factor)	10q11.2
PTC5*	GOLGA5	Golgi auto antigen	14q
PTC6*	hTIF1 α	Transcription intermediary factor	7q32-q34
PTC7*	hTIF1 γ RFG7	Transcription intermediary factor	1p13
PTC8*	Kinectin	Vesicle membrane anchored protein	14q22.1
PTC9*	RFG9	Putative Intracellular transport protein	18q21-22
RET/PCM1	PCM1	Centrosomal protein	8p22-p21.3
Elks-RET	ELKS	Unknown	12p13

* from patient exposed to Chernobyl radiations

Table 1 – RET oncogenes in papillary thyroid carcinomas

1.2.1.1 RET rearrangements as a common event

There are some common features that gather all RET fusion partner proteins. They are ubiquitously expressed, display cytoplasmic localization and contain different protein-protein interaction motifs. Coiled-coil domains often represent the dimerization/oligomerization domains present in the activating genes (Lupas et al, 1991; Lupas, 1996). Moreover, all the rearranged oncoproteins are able to self-associate, thanks to the oligomerization domain, and this triggers the constitutive trans-autophosphorylation of the tyrosine kinase domain, thus mimicking receptor dimerization upon ligand binding. The importance of promoting an oligomerization, which in turn leads to constitutive activation, has been demonstrated in several cases involving different oncogenes (ie RET/PTC1, RET/PTC2, RET/PTC3, TRK-T3, and also TPR/MET, Bcr/Abl, PLM/RAR) (Tong et al, 1997; Durick et al, 1995; Monaco et al 2001; Greco et al 1998; Rodrigues and Park, 1993; McWhirter et al, 1993; Grignani et al, 1999). Another common feature of these oncoproteins is a different sub cellular localisation with respect to the wild type receptors, which physiologically work on the cell surface. The chimeras are localised in the cytosol and this sub-localisation could be driven by the activating portion. In the case of RET/PTC2, different localization was observed for the two isoforms (Borrello et al, 2002). Finally, rearrangements

lead to the ectopic expression of kinases in epithelial follicular cells, although it has been recently demonstrated that RET could be physiologically active also in thyrocytes (Bunone et al, 2000).

All the chromosomal rearrangements are balanced reciprocal events. The rearrangements involve one of the two alleles of the activating gene. The non-rearranged allele is still present but the encoded protein could have a different sub cellular localization or its expression level could be decreased, as demonstrated for RET/PCM1 (Corvi et al, 2000). This could be either due to allelic inactivation or to the coexistence of wild type and rearranged forms that could affect protein function or stability. The haploinsufficiency could be directly responsible for tumorigenesis if the proteins involved in the oncogene activation are important for physiological cell activity (RI alpha for RET/PTC2).

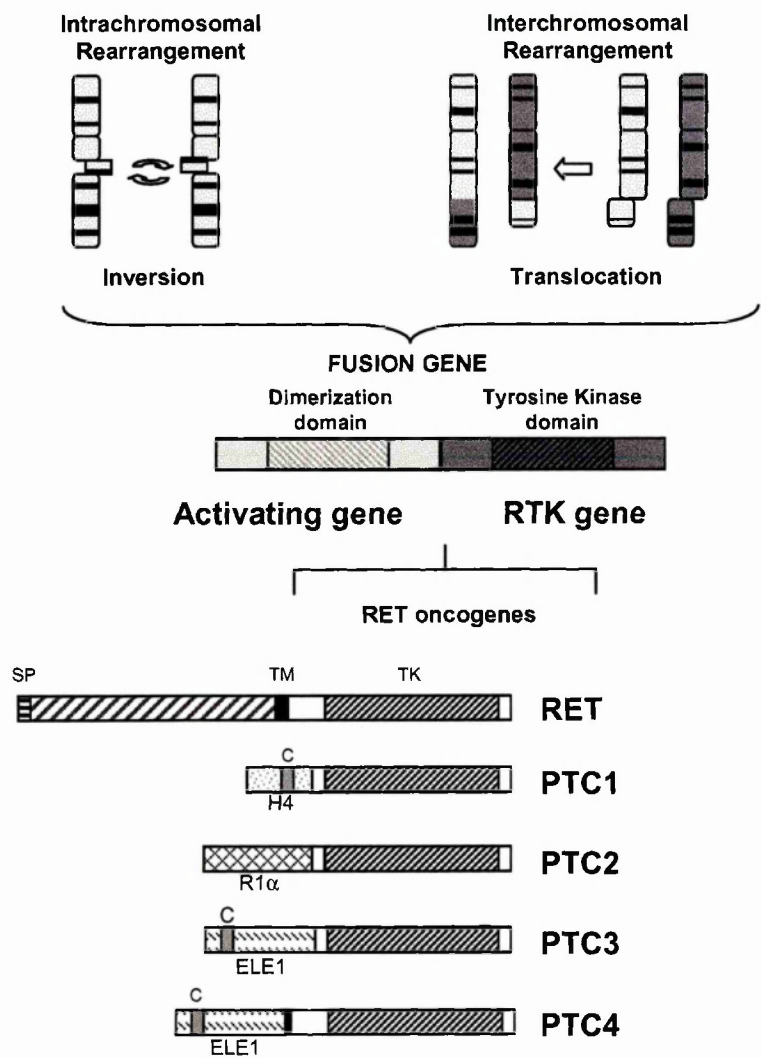


Figure 5. Mechanisms of chromosomal rearrangements generating fusion transforming genes. RET thyroid oncogenes.

These rearrangements cause the fusion of the RET tyrosine kinase encoding domain to the 5'-terminal domain of heterologous genes, such generating RET/PTC oncogenes. Common features of genes

activating RET: ubiquitous expression, cytoplasmic localization, presence of coiled-coil domains promoting dimerization Activating genes are indicated; SP: signal peptide; TM: transmembrane domain; TK: tyrosine kinase domain; C: coiled –coil domain.

1.2.1.2 Clinical features of PTC expressing RET oncogenes

Analysis of clinical characteristics of sporadic and radiation-induced papillary thyroid tumors indicates the existence of a correlation between RET positivity and young age of patients. In fact it has been reported that in papillary thyroid neoplasias, the frequency of RET activation is significantly higher in patients under the age of 30 (Bongarzone et al, 1996). In addition, children are much more sensitive to the tumorigenic effect of external irradiation, because of the high degree of replication of thyroid cells. This could amplify the possibility to fix and propagate mutational changes. More recently, controversial data (Elisei et al, 2001) suggested that RET/PTCs rearrangement in thyroid tumor is not restricted to the malignant phenotype, is not higher in radiation induced tumors compared with those naturally occurring, is not different after exposure to radioiodine or external radiation and is not dependent on young age.

The Chernobyl accident has increased the risk of childhood thyroid cancer. These tumors are more aggressive than sporadic

tumors and have an unusually short latency period between the exposure and the disease. Moreover several studies indicate a lower female to male ratio than sporadic thyroid tumors in children (Moysich et al, 2002).

Since many authors described RET rearrangements in occult PTCs (Viglietto et al, 1995) and papillary micro carcinomas (Nasir et al, 2000; Corvi et al, 2001), oncogene activation is considered an early event in thyroid carcinogenesis. This hypothesis is also supported by the evidence that transgenic mouse models of RET/PTC1 and RET/PTC3, driven by a thyroglobulin promoter, develop multifocal thyroid tumors at an early age, and the tumors are histologically very similar to human papillary thyroid carcinomas, with ground glass nuclei, grooves and inclusions (Santoro et al, 1996; Russell et al, 2000). However, RET/PTC1 transgenic mice infrequently developed solid-type carcinomas, differently from what observed in RET/PTC3 transgenic mice (Russell et al, 2000; Sagartz et al, 1997; Powell et al, 1998). In addition, the infection of short-term cultures of normal human thyroid cells with a retroviral vector expressing RET/PTC1 alters nuclear morphology with similar or identical changes diagnostic of papillary thyroid carcinoma (Fischer et al, 1998). Interestingly, the failure to identify RET/PTC in poorly differentiated and anaplastic thyroid carcinomas (Soares et al, 1998) the apparent inability of

papillary tumors harboring RET/PTC rearrangements to progress to less differentiated form (Tallini et al, 1998) and, on the contrary, the discovery of RET oncogene in transformed cells with apparent limited growth potential (Bond et al, 1994), point to a limited role for RET/PTC in the development of aggressive forms of thyroid cancer. Tallini et al. in 1998, analyzing 316 thyroid tumors concluded that only PTCs with alterations other than RET, e.g.: Trk gene, can progress to less differentiated, more malignant thyroid cancer. Recently, the same authors using immunohistochemical techniques, identify RET rearrangements also in a low % (12.9%) of poorly differentiated thyroid carcinomas (PDC) (Santoro et al, 2002) but the proportion raises to 20% among poorly differentiated thyroid carcinomas with evidence of evolution from a papillary thyroid cancer. This finding may suggest that PDC could derive from dedifferentiation of TPC but do not support a role for RET/PTC in the development of the more malignant phenotype.

1.2.1.3 Restriction of RET oncogenic rearrangements to PTCs

Thyroid carcinoma is the only adult epithelial malignancy where specific chromosomal rearrangements have been identified. Thyroid cancer is a rare malignant disease of the endocrine system and has been clearly linked to external ionizing radiation exposure. It has been

demonstrated that Chernobyl-related ionizing radiation exposure was directly related to the increased of developing papillary thyroid carcinoma risk in children from affected areas. Gene rearrangements involving the RET proto-oncogene, and less frequently NTRK1, have been shown to be causative events specific for papillary thyroid cancer (Bongarzone et al, 1996; Elisei, et al, 2001; Wajjwalku et al, 1992; Beimfohr et al, 1999). However, in experimental models, transforming ability of the RET/PTCs oncogenes is not restricted to the thyroid epithelium. In fact in the case of transgenic mice carrying RET/PTC1 gene under the control of the H4 promoter, Portella et al. report the developed of mammary adenocarcinomas, hyperplasia of sebaceous glands and pilomatrixomas (Portella et al, 1996). The specificity of these oncogenic rearrangements as peculiar feature for thyrocytes has been related to the higher frequency of proximity of the RET and H4 loci in interphase nuclei of human thyroid cells, compared to nuclei of peripheral blood lymphocytes and mammary epithelial cells. Spatial contiguity of RET and H4 suggests a structural basis for generation of RET/PTC1 rearrangement, because a single event could produce a double-strand break in each gene at the same site in the nucleus (Nikiforova et al, 2000a; Nikiforova et al, 2000b). This tendency is also proposed from the same author for RET/PTC3 rearrangement (Nikiforov et al, 1999). Moreover RET/PTC activation can be induced

by irradiation of human thyroid tissues implanted in mice (Mizuno et al, 2000) but not in irradiated mice thyroid, probably because the chromosomal architecture of mice chromosome carrying RET locus during interphase is different from that of human thyroid cells. This finding could explain the high level of RET rearrangements in patients irradiated for benign diseases or in children exposed to radiation after Chernobyl accident, where the incidence of RET/PTC activation ranges from 60-70% versus the 5-30% of the spontaneous papillary carcinomas. On the other hand Yang et al (Yang et al, 1997) observed that thyroid cells with DNA damage induced by exposure to ionizing radiation were resistant to apoptosis. In this context, the induced expression of wild type p53 might play an important role in promoting DNA end-jointing enzymatic activity in thyroid cells.

Follicular adenomas and carcinomas arise through an oncogenic pathway distinct from that of papillary carcinomas, characterized by a higher prevalence of activating mutations of all the three RAS genes and a greater predisposition to develop DNA copy abnormalities. In follicular carcinomas a fusion oncogene involving PAX8 and the peroxisome proliferator-activated receptor PPAR γ has recently been described. PAX8 encodes a paired domain transcription factor essential for thyroid development. Such rearrangement was not found either in follicular adenomas or in PTCs. The oncogene resulted from a

chromosomal translocation t(2;3) (q13;p25) in follicular tumors and displayed dominant negative suppression of wild type PPAR γ activities (Kroll et al, 2000).

In conclusion, a single thyrocyte can give rise to two different types of well-differentiated carcinomas, the papillary and the follicular. In this context the specific genetic alteration becomes crucial to drive the transformation of the thyrocytes towards the papillary (RET and NTRK1 rearrangements) or the follicular phenotype (PPAR γ rearrangements or RAS point mutations). It is possible that papillary carcinomas lacking RET or TRK activation may carry rearrangements of other tyrosine kinase receptors rather than defects in genes involved in RET and TRK signaling pathways.

Finally it is worth mentioning that a few cases of familial papillary thyroid carcinomas (FPTCs) have been recently described although its mode of inheritance as well as its genetic and molecular bases are still poorly understood. No linkage to RET had been found in these tumors until Corvi (Corvi et al, 2001) demonstrated that one FPTC and the adenoma from the same patient carry a RET rearrangement (type PTC1) and that this rearrangement is absent in the germline. Furthermore, they excluded a RET haplotype sharing in two brothers of the same family. These results show that RET rearrangements can indeed be found in FPTC and confirm that RET is

not involved in the inherited predisposition to FPTC. On the other hand, McKay (McKay et al, 2001) recently indicated the existence of a susceptibility locus for familial NMTC (a complex genetic disorder characterized by multifocal neoplasia and in particular by papillary thyroid carcinoma with a higher degree of aggressiveness than its sporadic counterpart) on chromosome 2q21.

1.2.2 RET activation in inherited and sporadic medullary thyroid carcinomas

The C cells of the thyroid are derived from the neural crest and are believed to be the precursors from which medullary thyroid carcinoma (MTC) arises. As many as 75% of all MTCs are sporadic; the remaining hereditary forms of MTC are associated with Multiple Endocrine Neoplasia type 2 (MEN2) which was the first of the inherited endocrine neoplasia syndromes to be elucidated at the genetic level with the discovery in 1993 that germline mutations in the RET proto-oncogene were present in affected individuals (Mulligan et al, 1993). Unlike other cancer syndromes, which are associated with inactivation of tumor suppressor genes, MEN2 arises as a result of activating mutations of the RET proto-oncogene.

Germline point mutations of RET are responsible for the inheritance of MEN2 cancer syndromes which are usually divided into three different

clinical subtypes: MEN2A, MEN2B and FMTC, all autosomal dominant cancer syndromes.

The MEN2A subtype is characterised by MTC, pheochromocytoma, and parathyroid hyperplasia (Eng et al, 1996; Mulligan et al, 1995) whereas MTC, pheochromocytoma, ganglioneuromas of the intestinal tract and skeletal and ocular abnormalities characterise MEN2B. MEN2B is the most aggressive of the three sub-types, often displaying an earlier age of onset.

MTC is the only feature of FMTC and it usually develops at a later stage of life. The course of MTC in FMTC families is more benign and prognosis is good (Famdon et al, 1986).

1.2.2.1 Medullary thyroid carcinoma

MTCs arise from thyroid C cells which are named for their ability to secrete calcitonin. Calcitonin is produced by MTCs and is useful as a specific MTC tumor marker. MTCs are well demarcated, firm, gray-white tumors that may have a gritty consistency due to calcifications. Almost all hereditary MTCs are bilateral or multifocal, and only 6% are solitary.

MTC spreads within the central compartment to pre tracheal lymph nodes (Level VI nodes), and frequently to upper mediastinal nodes (Level VII nodes). Further lymphatic spread can also occur to the

lateral neck compartment, including the jugular (Levels II, II, and IV nodes), posterior triangle (Level V nodes), and supraclavicular nodes. Primary MTC and lymph node metastases can invade or compress adjacent structures, most commonly the trachea, recurrent laryngeal nerve, jugular veins and carotid arteries. Distant metastases typically occur in the liver, lungs and bone and can be associated with small primaries. Patients who are diagnosed with MTC before it is palpable, rarely have lymph node metastases. In contrast, most patients with sporadic MTC, or with hereditary MTC not detected by genetic screening, display a neck mass and have a very high rate of lymph node involvement (reviewed in Phay et al, 2000).

1.2.2.2 Multiple endocrine neoplasia type 2A (MEN2A)

In virtually all MEN2A, mutations affect the RET cysteine-rich extra cellular domain, each converting a cysteine to another amino acid, at codons 630, 634 (exon 11) or codons 609, 611, 618, 620, (exon 10). These mutations account for 98% of all the mutations associated with MEN2A; the most common mutation accounting for over 80% of all mutations associated with MEN2A affects codon 634 and converts a cysteine into an arginine. Rarer mutations associated with MEN2A have been described such as an in-frame germline duplication of 12 and 9 bp in exon 11 (Hoppner and Ritter, 1997;

Hoppner et al, 1998) and de novo cases of MEN2A have been associated with two new germline mutations (at codons 634 and 640) on the same RET allele (Tessitore et al, 1999) or at codon 624 (Aguild, 1999). RETMEN2A oncoproteins display constitutive kinase activity due to ligand-independent dimerization. The substitution of one cysteine residue, leads to constitutive receptor dimerization and hence activation as the loss of an intramolecular disulphide bond results in an unpaired cysteine residue available to take part in an intermolecular disulphide bond between two mutant RET receptors (Asai et al, 1995; Santoro et al, 1995; Borrello et al, 1995). Consistent with this, transgenic mice in which the C634R mutation in the ret gene coding for the short isoform was expressed under the control of the human calcitonin promoter or under the MoMuLv LTR, develop C-cell tumors resembling human MTC (Michiels et al, 1997; Kawai et al, 2000). Interestingly, transgenic mice expressing the same mutation in the RET long isoform, developed both MTCs and papillary thyroid carcinoma (Reynolds et al, 2001).

1.2.2.3 Multiple endocrine neoplasia type 2B (MEN2B)

Most MEN2B cases (95%) are caused by the M918T mutation (exon 16) that is frequently a de novo mutation located on the allele inherited from the patient's father (Carlson et al, 1994a). Other rarer

(5%) intracellular mutations involve codon 883 (exon 15) in the RET tyrosine kinase domain. The M918T substitution is also found in sporadic MTC, with M918T mutation-positive tumours often displaying a more aggressive phenotype. Recently, infrequent germline missense mutations have been reported in MEN2B *de novo* cases: in exon 16 at codons 912 and 922 (Carlson et al, 1994b). Moreover, a double mutation at codons 804 and 806 has been found in a Japanese patient that had clinical features characteristic of MEN2B (Miyachi et al, 1999). The reason why more than 95% of MEN2B cases are accounted by germline M918T and only fewer than 5% by A883F is unknown. The M918T mutation does not cause constitutive dimerization although GDNF stimulation seems to be necessary for the full activation of the MEN2B mutant RET (Bongarzone et al, 1998), and the overall activation levels of the RET kinase it induces can hardly account for its high oncogenic potential (Borrello et al 1995; Santoro et al, 1995). Thus, RETMEN2B is probably more than simply an active RET kinase, and qualitative changes in RET kinase activity may be responsible for its specific neoplastic phenotype. RET methionine at codon 918 is highly conserved in receptor tyrosine kinases, and it maps in a loop of the kinase domain that is predicted to interact with the protein substrate. A threonine is found at the equivalent position in cytosolic tyrosine kinases, and the two kinase classes (receptorial and cytosolic)

have different signaling specificities (Marengere et al, 1994). Accordingly, the MEN2B mutation converts the substrate-binding pocket of RET to resemble that of non-receptor tyrosine kinases such as c-src and c-abl (Carlson et al, 1994b; Hofstra et al, 1994). The change in substrate specificity can affect RET-mediated phosphorylation of intracellular proteins as well as the pattern of RET autophosphorylation sites. Both possibilities have been experimentally proven. The pattern of phosphorylated intracellular proteins differs in RETMEN2B- and RETMEN2A-expressing cells (Santoro et al, 1995). Moreover, phosphopeptide mapping has shown that RETMEN2B autophosphorylation sites differ from those of wild-type RET and of RETMEN2A (Santoro et al, 1995; Liu et al, 1996; Murakami et al, 2002). Thus, the shift of RET autophosphorylation sites and of RET intracellular substrates, rather than the simple raise of RET kinase activity, may be crucial for the oncogenic activity of RETMEN2B alleles. It is not known how the A883F affects RET function. However, residue 883 is located in a subdomain of RET that defines substrate preference (Smith et al, 1997) thus suggesting that the alteration of substrate specificity may be the common etiologic thread that underlies the pathogenesis of MEN2B. The production of a mouse model of MEN2B by introduction of the corresponding mutation into the *ret* gene demonstrated that heterozygous mutant mice displayed several

features of the human disease, including C-cell hyperplasia progressing to pheochromocytoma, while homozygous displayed more severe thyroid adrenal disease as well as male infertility. Only homozygous mice developed ganglioneuromas of the adrenal medulla and enlargement of the associated sympathetic ganglia (Smith-Hicks et al, 2000).

1.2.2.4 Tissues affected in MEN2

Thyroid C cells

The biological role of thyroid C cells is the production of calcitonin, a peptide hormone, and its secretion in response to raised blood calcium levels. Calcitonin then acts to lower blood calcium levels by increasing uptake into bone. C cells also produce a number of other peptide hormones, but the biological significance of these is not clear (Scopsi, 1991). The neural crest origin of at least some thyroid C cells was shown by grafting the quail neural tube into chickens (Le Douarin, 1970). In mammals, the vagal neural crest progenitors of C cells migrate into the ectoderm of the fourth pharyngeal pouch, forming the ultimobranchial body. At around 12.5 days post coitum (dpc) in the mouse, the ultimobranchial bodies fuse with the thyroid diverticulum, which is formed from an outgrowth of the endoderm from the back of the tongue which bifurcates around the trachea (Thiler et al, 1989). At

birth, the ultimobranchial component is indistinguishable as a separate entity from the thyroid diverticulum component, with C cells dispersed in parafollicular positions between thyroid follicles.

Although it is generally accepted that C cells are neural crest-derived, some authors argue that at least some C cells may have an alternative, endodermal origin. The strongest evidence to support this claim is the existence of mixed medullary-follicular tumours which are immunopositive for both follicular cell markers, such as thyroglobulin, and neuroendocrine markers such as calcitonin gene related peptide (CGRP), chromogranin A and carcinoembryonic antigen (Holm et al, 1987; Ljungberg et al, 1983; Hales et al, 1982; Marth et al, 1996).

Moreover, the precise lineage relationships between C cells and other neural crest derivatives is unknown and similar biochemical properties to enteric neurons and their origin from vagal neural crest, suggest that they are more closely related to gut neuroblasts than to adrenal chromaffin cells, which arise from multipotent cells of the trunk neural crest.

Studies on RET expression have also suggested that the C cell population may be heterogeneous. In the postnatal rat, a small number of C cells were said to be immunopositive for RET, although it was not reported whether developing C cells in the embryo were also positive (Tsuzuki et al, 1995). Similarly, *in situ* hybridisation of RET

gave a signal in only a subpopulation of human C cells (Fabien et al, 1994). Nevertheless, it is possible that all C cells express RET, but that in the majority of them it is below the threshold of detection.

The role of RET in normal thyroid C cells is not clear. In fact, RET null mice appear to have a normal thyroid C cell population (Pachnis, unpublished data). This suggests that RET is not directly involved in thyroid C cell development, despite the fact that it is expressed in the neuroectodermal lineage in the pharyngeal arch region at 8.5-9.5 days post coitum (Pachnis et al, 1993). The development of other vagal crest derivatives (mid and hindgut enteric neurons and the superior cervical ganglion) is RET-dependent (Durbec et al, 1996).

The adrenal chromaffin cells

Chromaffin cells of the adrenal medulla are derived from the trunk neural crest which also gives rise to the sympathetic ganglia (Anderson et al, 1993) and foregut enteric neurons (Durbec et al, 1996). As with C cells, there is no apparent defect in the adrenal medulla in RET knockout mice, suggesting that RET is not directly involved in their formation. The development of foregut enteric neurons is also RET-independent (Durbec et al, 1996). Although RET expression is detected by in situ hybridization in the sympathetic ganglia at 14.5 days post coitum in the mouse embryo, no expression

can be detected in embryonic adrenal chromaffin cells (Pachnis et al, 1993). Only isolated chromaffin cells show RET immunopositivity in the adult rat (Tsuzuki et al, 1995). Human pheochromocytomas show relatively strong expression of RET mRNA even though the signal obtained from chromaffin cells is weak (Santoro et al, 1990).

The parathyroid gland

Parathyroid glands are endocrine structures which release parathyroid hormone in response to depleted levels of blood calcium. These structures are derived from the endoderm of the third branchial pouch (Manley and Capecchi, 1995): In the developing embryo, RET is expressed in this region of endoderm but its role is unknown.

1.2.2.5 Familial medullary thyroid carcinoma (FMTC)

FMTC mutations can be found either in the extracellular or in the tyrosine kinase domain. The ones occurring in the extracellular domain of RET are usually a set of substitutions of cysteines 609, 611, 618, 620 (exon 10) and both 630 and 634 which are also found associated with MEN2A, whereas in the tyrosine kinase domain, mutations occur at residues 768, 790, 791 (exon 13), 804, 844 (exon 14) or 891 (exon 15). Rare mutations have been recently reported, such as a 9-base pair duplication in exon 8 in an FMTC family (Pigny et al, 1999) or

mutations at codons 804 and 778 on the same RET allele which are associated with both FMTC and prominent corneal nerves (Kasprzak et al, 2001). FMTC mutations occurring in the intracellular RET domain were thought to be infrequent and only a small number of families bearing a RET mutation within exons 13, 14 and 15 had been described. In the past two years the frequency of detection of these mutations has increased (Niccoli-Sire et al, 2001) due to more accurate analysis and screening. Of note, mutations in exon 13 at codons 790 and 791 and in exon 14 at codon 804, until recently associated with the FMTC phenotype, have also been found in MEN2A. Cysteine mutations in the RET extracellular domain sometimes induce the FMTC phenotype and in contrast with what happens in MEN2A families, the substitution at codon 634 occurs only in 30% of the cases whereas mutations at codons 609, 611, 618, 620, 630, occurred in 60% of the FMTC families (Ponder and Smith, 1996). The transforming activity of RET with a substitution in cysteine 609, 611, 618, 620 or 630 was significantly lower compared to that of RET with the cysteine substitution at codon 634. This low transforming activity could then favour the development of FMTC rather than MEN2A.

No data are yet available on the mechanisms of activation of FMTC mutations occurring in the RET tyrosine kinase domain. Patients with RET mutations in exons 13, 14 and 15 exhibit a mild C cell

disease phenotype (Berndt et al, 1998; Fattoruso et al, 1998) confirmed by *in vitro* studies. In fact, mutants expressing E768, V804 and S891 RET mutations display lower transforming activity (Pasini et al, 1997; Iwashita et al, 1999) compared to RET substitutions at codons 634, 918 or 883, strongly associated with MEN2A and MEN2B respectively. Computer modelling has suggested that the E768D substitution modifies the kinase activity of the receptor by altering substrate specificity or ATP-binding capacity (Pasini et al, 1997). As for its location, also the substitution at position 804 may exert an activating effect by altering the kinetics of interactions with normal cellular substrates or by modifying the range of substrates that are phosphorylated (Bolino et al, 1995; Eng et al, 1995; Iwashita, et al, 1999; Pasini et al, 1997).

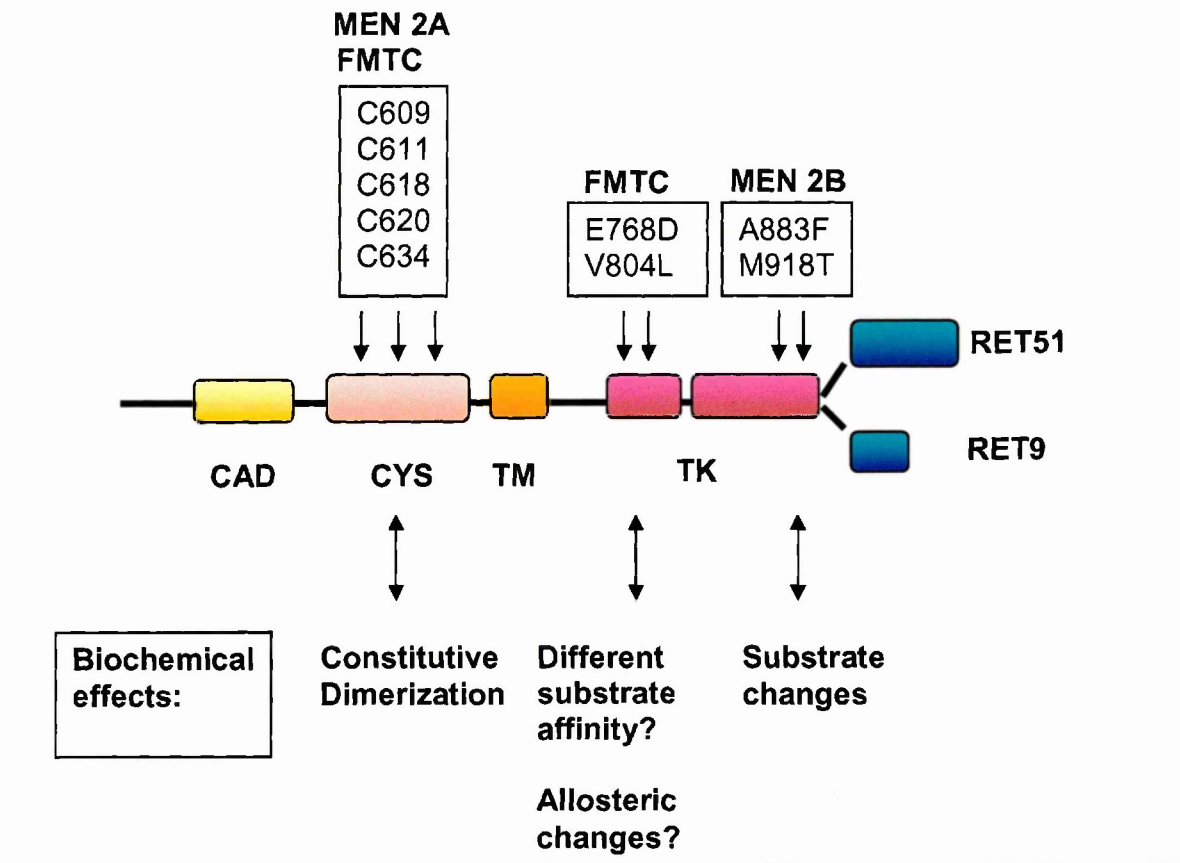


Figure 6. RET oncogenic mutations. Activating germline mutations of RET are responsible for the MEN2A syndrome which comprises three different diseases: MEN2A, MEN2B and FMTC.

1.2.2.6 Sporadic MTC

MTC is sporadic in 75% of all cases and hereditary in the rest. Hereditary MTC is well defined: germline gain-of-function mutations in

the *RET* proto-oncogene cause multiple endocrine neoplasia type 2 (MEN 2) (Eng et al, 1996a). However, the majority of cases are sporadic. The aetiology of sporadic MTC, similar to most neuroendocrine tumours, remains elusive. Although LOH at various loci (Mulligan et al, 1993), and somatic mutations in *RET*, mainly at codon 918, occur in MTC (Hofstra et al, 1994; Eng et al, 1995; Eng et al, 1996a, 1996b, 1999), the aetiology and pathogenesis of these sporadic tumours remains largely unknown. Somatic *RET* mutations are found in up to 70% (mean 30–50%) of DNA from sporadic tumours (Eng and Mulligan, 1997). These somatic mutations are often heterogeneously present in tumour DNA, indicating that they more likely occur during clonal evolution rather than presenting the initial step of carcinogenesis. Deletions of several chromosome arms (1p, 3p, 3q, 11p, 13q, and 22q) have been reported in up to 38% (Mulligan et al, 1993). No tumor suppressor gene has been identified yet. Recently, over-representation of a germline polymorphic sequence variant located within exon 14 of the *RET* proto-oncogene at codon 836 (nt C, 2439C to T, S836S) was found to be associated with sporadic MTC, with a relative risk of approximately 3 (Gimm et al, 1999).

Mutations at cysteine codons 609, 611, 618, 620, 630, 634 appear also in sporadic MTC cases and recently three new somatic missense mutations (at codons 639, 641 and 922) of the *RET* proto-

oncogene associated with sporadic MTC have been described (Kalimin et al, 2001).

Recent studies (Feldman et al, 2000; Brauckhoff et al, 2002) have reported cases of patients harboring RET germline mutations in exons 14 and 15 (at codons 790, 791, 804) resulting in papillary micro carcinoma. Moreover, Rey et al. (Rey et al, 2001) also described the case of a kindred in which a novel single point germline RET mutation (K603E in exon 10) co segregates with medullary and papillary thyroid carcinomas. Despite the low number, these observations suggest that there might be a correlation between the occurrence of PTC and RET germline mutations in exons 13 and 14 that may play a role in the pathogenesis of PTC. Of note, PTC seems to be present only in patients with low penetrance RET germline mutations. It remains an open question whether the simultaneous occurrence of inherited MTC and PTC is coincidental or the result of partly common pathogenic pathways. Reynolds et al. (Reynolds et al, 2001) found the co-existence of MTC and PTC in transgenic mice expressing the long isoform of MEN2A RET and suggested that this might be due to the possible existence of an ultimobranchial stem cell of endodermal origin, which gives rise to a subset of both thyroid follicular cells and C-cells (Kovacs et al, 1994).

In conclusion, the genotype/phenotype associations in MEN2 reflect differences in behavior and function among the RET mutant forms. The above data suggest a model in which there are tissue specific differences in sensitivity to RET activation. All RET activating mutations in fact, are sufficient to induce tumorigenesis in the thyroid glands and MTC is associated with all MEN2 subtypes. On the other hand, pheochromocytoma and hyperparathyroidism are mainly found in association with the most penetrant of RET mutations affecting cysteine 634, suggesting that high transforming activity is required to induce abnormal growth in these tissues.

1.2.2.7 Diagnosis and management of MEN2

The prognosis for MEN2 patients is very good with early diagnosis and intervention, thus implying that adequate testing is required to screen subjects at risk for MTC. Currently, early genetic screening for RET mutations is considered the standard care for MEN2, since patients having a MEN2/FMTC-specific germline mutation have a high risk of developing MTC. Prophylactic thyroidectomy is recommended before the age of 6 years (reviewed in Gimm et al, 2001) as every single C-cell inherits the genetically determined potential to become neoplastic. Clinical research concentrates on finding an existing genotype-phenotype correlation, mutations which

are associated with a late age of onset, that would allow postponing surgery, thus preserving the thyroid until adulthood or restricting the extent of surgery. Several mutations such as E768D and V804M seem to qualify for such a recommendation (Gimm et al, 2001). MEN2B-associated MTC is the most aggressive and early diagnosis is required and total thyroidectomy is recommended as early as 1 year. Given that even low penetrance RET mutations, particularly codon 804 mutations, might exist in MEN2, it might be wise for all individuals who present MTC to undergo RET testing. The discovery of an occult germline RET mutation in an apparently sporadic MTC case means that the individual has MEN2. In order to simplify the detection of RET missense mutations, in a very recent paper (Kim et al, 2002), a method for the rapid mutation analysis of gene sequences has been proposed using oligonucleotide microarrays instead of the commonly used analysis of RET mutations by single-strand conformational polymorphism or direct sequencing. This might represent an effective diagnostic genetic tool to test for the presence of MEN2 mutations at early stages.

As MTC respond very poorly to chemotherapeutic agents and total thyroidectomy is the only way to treat these tumors, the availability of inhibitors specific for RET oncoproteins could help in developing new therapeutic strategies for RET-associated diseases.

Carlomagno et al. (Carlomagno et al, 2002) have very recently demonstrated that the pyrazolo-pyrimidine PP1 blocks tumorigenesis induced by RET/PTC cytoplasmic oncogenes by inhibiting RET enzymatic activity and thus its transforming effects. Our results as well, suggest that PP1 treatment would represent a promising new strategy to selectively target RET oncogenic products for destruction holding promise for MTC therapy.

1.2.3 RET germline inactivating mutations in Hirschprung disease

Hirschsprung disease (HSCR), or colonic aganglionosis, is a common congenital disorder (one in 5000 live births) leading to intestinal obstruction or chronic constipation (Parisi and Kapur, 2000). Most HSCR cases are sporadic, however 15-20% are familial forms and genetic analysis has identified mutations in pathways related to RET, endothelin and in two transcription factors, Sox 1 and the SMAD-interacting protein-1 (SIP1) (Parisi and Kapur, 2000; Wakamatsu et al, 2001; Cacheux et al, 2001). Heterozygous mutations of GDNF and NTRN have also been identified in a small fraction of HSCR patients (Parisi and Kapur, 2000) but no mutations have been described for RET coreceptors (Angrist et al, 1998; Myers et al, 1999; Onochie et al, 2000; Vanhorne et al, 2001) even though Gfra α 1 knockout mice are phenotypically very similar to RET and GDNF -/- mice (Cacalano et al,

1998; Enomoto et al, 1998). However, RET seems to be the major gene involved in HSCR; even when the major mutation is in the endothelin receptor B (EDNRB) gene (Puffenberger et al, 1994), RET variants make some contribution to susceptibility and homozygous Ret-null mice have full sex-independent penetrance of aganglionosis (Schuchardt et al, 1994). Moreover, even the mutations affecting RET ligands are not sufficient by themselves to induce the HSCR phenotype, but contribute to the disease when associated with other mutations in RET (Parisi and Kapur, 2000).

Inactivating mutations of one allele of the RET proto-oncogene have been detected in half of the dominantly inherited cases of HSCR that displays incomplete penetrance, and in one third of HSCR sporadic cases (Eng, 1996; Eng and Mulligan, 1997). RET mutations are spread throughout the coding sequence and include deletion, insertion, frame shift, nonsense and missense mutations (Eng and Mulligan, 1997; Parisi and Kapur, 2000). Most of these mutations impair RET function (Parisi and Kapur, 2000; Iwashita et al, 2001), thus suggesting that HSCR results from RET haploinsufficiency, but the allelic heterogeneity at the RET locus in HSCR is associated with various mechanisms of action leading to RET dysfunction (Pelet et al, 1998; Iwashita et al, 2001). Mutations located within the extracytoplasmic domain impair RET maturation and its translocation to the plasma membrane

(Carlomagno et al, 1996; Iwashita et al, 1996). This probably results in a decrease in the amount of the RET protein at the cell surface, which is insufficient to trigger RET signalling in enteric neuroblasts during embryogenesis. HSCR mutations in the RET tyrosine kinase domain affect the catalytic activity to different extent. Some of them completely abolish the receptor kinase activity, others interfere with the binding of components of RET signalling such as PLC- γ for the E762Q substitution (Iwashita et al, 2001) Shc, IRS-2 or FRS2 (Geneste et al, 1999; Melillo et al, 2001b; Melillo et al, 2001a; Bordeaux et al, 2000). Recently, Bordeaux et al. (Bordeaux et al, 2000) suggested that RET can induce caspase dependent apoptosis in the absence of its ligand. HSCR may then result from apoptosis of RET-expressing enteric neuroblasts since HSCR mutations render this proapoptotic activity ligand insensitive.

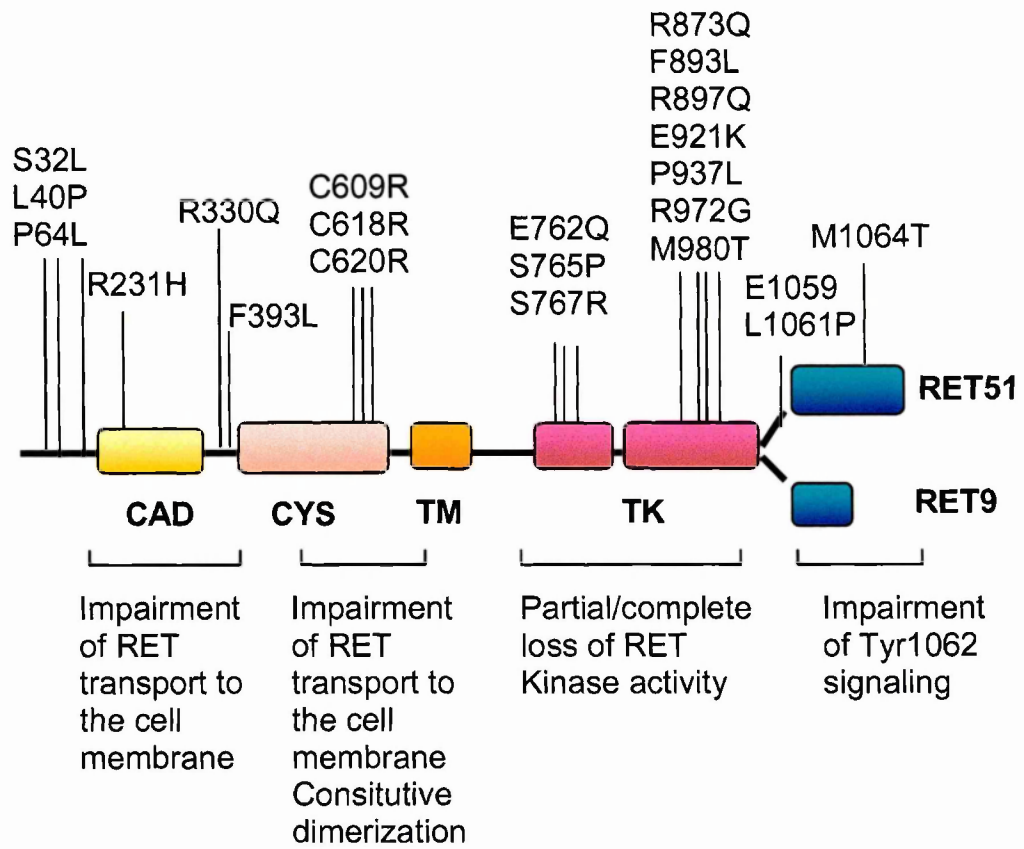


Figure 7. RET inactivating mutations. Inactivating mutations of RET cause the impaired development of the enteric nervous system which is responsible for the Congenital megacolon or Hirschprung's disease (HSCR)

1.2.4 Co segregation of MEN2A/FMTC and HSCR

In rare families, MEN2A/FMTC and HSCR co-segregate (Mulligan et al, 1994; Decker et al, 1998; Takahashi et al, 1999) and affected individuals carry a single substitution at one of the four cysteines in the extra cellular RET domain (codons 609, 611, 618 and 620). The reason why a single mutation can display antagonistic effects is still under current studies. It is possible to speculate that this is due to the differences in the timing of RET expression in each particular tissue that might differently affect tissue development in neural-crest derived cells. These mutations result, in uncontrolled cellular proliferation in endocrine tissues and on the other hand, result in a lack of neural growth in the enteric system (Takahashi et al, 1999). Genetic or environmental factors might also influence the clinical expression of the enteric phenotype. In endocrine tissues in fact, these cysteine substitutions have a dominant effect, whereas it is not yet clear if the loss or inactivation of the wild type RET allele, in the enteric system, is associated with the HSCR phenotype.

Experimental data demonstrated that RET MEN2A/HSCR mutations markedly decrease RET cell surface expression (Ito et al, 1997) resembling the typical HSCR mutations of the RET extra cellular domain. Interestingly, both transfection experiments and biochemical analysis have shown that the cysteine codon substitutions were also

able to cause ligand independent dimerisation, activation and transformation as do the classical MEN2A cysteine substitutions (Asai et al, 1995; Santoro et al, 1995; Borrello et al, 1995), although to different extents. Since these mutations trigger the development of medullary thyroid carcinoma, as well as pheochromocytoma, the mislocalization of the receptors affect pathways not involved in cell proliferation but probably controlling other pathways such as cell movement and migration, probably essential for neuronal development.

Disease	Genetic aiteration	Pathogenic mechanism
	RET	
PTCs	chromosomal rearrangements	constitutive TK activity
MEN2A	germline point mutations in the cysteine-rich domain	constitutive disulphide linked dimerization
MEN2B	germline point mutations in RET TK domain	constitutive TK activity altered substrate specificity
FMTC	germline point mutations - in the cysteine-rich domain - in RET TK domain	constitutive dimerization constitutive TK activity? altered substrate specificity ?
HSCR	germline point, frame shift, missense mutations in: - RET extracellular domain - RET tyrosine kinase domain - RET COOH terminus	impairment of RET cell surface expression partial or total loss of RET TK activity impairment of binding of docking proteins

Table 2. Involvement of RET in human pathologies

1.3 Aim of the present study

The aim of the work in the following chapters was to study of the expression of different RET mutants in order to highlight their biological role in diverse cellular contexts. In particular, we focused on gain of function cysteine mutations that are responsible for medullary thyroid carcinoma (MTC) by causing the covalent dimerization of RET, leading to ligand-independent activation of its tyrosine kinase. In this context, the association of Cys⁶⁰⁹, Cys⁶²⁰ and activating mutations with HSCR is still an unresolved paradox. To address this issue, we have developed a transgenic model for human diseases (specifically, Multiple Endocrine Neoplasia type 2 and Hirschsprung disease) through the insertion of a gain and loss of function RET mutation, the RET^{C620R} into the mouse genome.

Since a mouse model of MTC which reflects the human tumour could be used for studies aimed at developing therapeutic strategies for carcinomas sustaining RET activation, we have also studied the *in vitro* effects of a tyrosine kinase inhibitor PP1, that we propose as possibly representing a potential treatment strategy and thus meriting testing using *in vivo* models such as the one we have generated.

2. MATERIALS AND METHODS

2.1 Cell Culture

Mouse fibroblast NIH3T3-untransfected cells were grown in DMEM containing 10% calf serum whereas NIH3T3-transfected cells were grown in DMEM containing 5% fetal calf serum (FCS). HEK 293T cells were grown in DMEM supplemented with 10% FCS. The human TT cell line was cultured in Ham's F-12 (Life Technologies, Inc.) supplemented with 15% FCS. SK-N-MC neuroepithelioma cells were grown in DMEM containing 10% FCS. The MDA-MB-231 cell line was cultured in RPMI supplemented with 10% FCS.

2.2 Maintenance of cell cultures

Culture media were made fresh every 7 days. Adherent cells were split using 0.25% trypsin/EDTA (once detached, the cells were mixed with an equal volume of growth medium to inactivate the trypsin) and harvested by centrifugation at 1000 rpm for 10 minutes. The cell pellet was resuspended in growth medium and an aliquot was diluted in trypan blue for counting. Only viable cells (those that excluded trypan blue) were counted.

2.3 Storage of cell lines in liquid nitrogen

Stocks of cells were kept under liquid nitrogen at a density of 1×10^6 cells/ml in growth medium containing 30% serum and

10% (v/v) dimethyl sulfoxide (DMSO-Sigma cell culture). 1 ml aliquots of the cell suspension were aliquoted into Nunc™ cryovials and the vials were frozen overnight at -80°C before transferring to liquid nitrogen for long term storage.

Cells were removed from liquid nitrogen when required and thawed rapidly. The cell suspension was diluted to 10 ml in proliferation medium and washed 3 times by centrifugation to remove the DMSO before seeding in growth medium.

2.4 Preparation of expression constructs

2.4.1 Sub-cloning into expression vectors

Before transfection, each cDNA was subcloned into the suitable expression vector, pcDNA.3 (Invitrogen). cDNAs were excised with the appropriate restriction enzymes and purified from agarose gel slices using the Qiagen™ Qiaquick gel extraction kit, according to manufacturer's instructions. Vectors were restricted, the termini dephosphorylated and purified for ligation. Ligations were carried out at 15°C overnight using T4 ligase (Boehringer Mannheim), according to manufacturer's instructions.

2.4.2 Restriction enzyme digestion of DNA

Reactions were carried out in 20µl volume following the protocols of Maniatis et al 1993. Loading buffer was added to the digest prior to electrophoresis. Restriction fragments were visualised by

electrophoresis on agarose 1X TBE buffered gels stained with 2.5ng ethidium bromide.

2.4.3 Transformation of competent bacteria with expression vectors

2.4.3.1 Preparation of competent bacteria

E.coli, strains HB101 and JM109, stored in 300ml aliquots at -80°C were spread onto a Luria Bertani (LB) agar plate and grown overnight at 37°C . The same day, a single colony of bacteria was picked and used to inoculate 6ml of LB medium in a 15ml tube (Falcon) and grown overnight at 37°C with shaking at approximately 250 rpm. 4ml of the overnight culture were transferred into a 1l conical flask with a loose cap containing 400ml of LB medium. The culture was incubated for 2 hours at 37°C with shaking at approximately 250 rpm. After 2 hours. Zero point five ml samples of the culture were taken at 15 minute intervals and the optical density (O.D.) at 590nm measured. When the O.D. was between 0.3 and 0.375, the culture was judged to be in a log phase of growth and transferred to 50ml Falcon tubes which were cooled on ice for 10 minutes. Bacteria were collected by centrifugation at 2500rpm for 12 minutes at 4°C . the medium was discarded and each pellet was carefully re-suspended in 10ml of ice-cold, autoclaved 100mM calcium chloride, pooled and kept in ice for at least 30 minutes to

increase the efficiency of transformation. 0.45ml 80% glycerol was added to the suspension to give an 8% final glycerol concentration. The competent cells were divided into 300µl aliquots and immediately frozen in dry ice before storing at -80°C.

2.4.3.2 Transformation of competent bacteria

E. coli bacteria were transformed by heat shock. 17.4µl of 2-mercaptoethanol was added to 1ml of sterile H₂O the solution was mixed and cooled on ice. An aliquot of frozen competent cells were thawed on ice, added to 10µl of the cold 2-mercaptoethanol solution in a sterile 15ml round bottomed tube and left on ice for 3 minutes. Approximately 20ng of DNA was added, the tubes were tightly capped, and left on ice for 40 minutes. The bacterial suspension was heat shocked by placing it in a 42°C water bath for 2 minutes and then cooled on ice. 1ml of LB broth (pre-warmed to 37°C) was added and the bacteria were incubated in aerated tubes for 1 hour at 37°C with shaking at approximately 250rpm. Aliquots of transformed cultures were plated onto LB agar plates containing 100µg/ml ampicillin and grown overnight at 37°C. Individual colonies were selected and analysed by alkaline mini-preparations of plasmid DNA, which were mapped by restriction enzyme digestion to identify clones

containing the required, single copy insert in the correct orientation.

2.4.4 Alkaline mini preparations of plasmid DNA

Individual preparations of colonies were inoculated into 4ml of LB broth containing 100µg/ml ampicillin and incubated overnight at 37°C with shaking. 1.5ml of the overnight culture were transferred into a 1.5ml microcentrifuge tube and bacteria were collected by centrifugation at 5000rpm for 5 minutes in a microcentrifuge. The remainder of each culture was stored at 4°C. The supernatant was removed and the bacteria were resuspended in 100µl glucose/tris/EDTA (25mMTris/HCl pH8.0, 10 mM EDTA pH8.0, 50mM glucose). The bacteria were lysed by the addition of 200µl of 0.2M NaOH containing 1% SDS. The contents of the tube were mixed and put on ice for 5 minutes. 150µl of 5M potassium acetate was added to the lysate, which was incubated on ice for a further 10 minutes. After mixing, the precipitate was collected by centrifugation in a microcentrifuge at 13000 rpm for 10 minutes at room temperature. The supernatant was transferred to a new microcentrifuge tube and the plasmid was precipitated for 1 hour at -20°C after addition of 10% (v/v) sodium acetate pH5.2 and two volumes of 100% ethanol, pre-cooled to -20°C. The precipitate collected by

centrifugation was washed twice with ethanol 70%, pre-cooled to -20°C . The ethanol was discarded and without drying, the pellet was re-suspended in 100 μl TE buffer (10mM Tris.HCl pH 8.0 1mM EDTA pH 8.0). RNA was precipitated adding 100 μl lithium chloride, vortexing and centrifugating at 13000rpm for 10 minutes in a bench-top microcentrifuge to bring down the lithium-rRNA precipitate. The supernatant was transferred to a new microcentrifuge tube and DNA was precipitated and washed with ethanol 70%. The final pellet was dried and resuspended in TE buffer.

2.4.5 Maxi preparations of plasmid DNA

Colonies of bacteria which contained the expression vector containing a single copy of the required insert in the correct orientation were chosen for maxi-preparations of plasmid DNA using Qiagen Plasmid Maxi Kit with Qiagen tip 500, according to manufacturers instructions. DNA was resuspended in TE buffer pH 8.0. Purity was checked by spectrophotometry and integrity of the construct was assessed by restriction enzyme digestion.

2.5 Transfection and cloning of mammalian cell lines

NIH3T3, SKNMC and 293T cells were used in transfection studies. Transient transfections were carried out using either the lipofectamine or calcium phosphate methods. Lipofectamine was

used according to the manufacturer' instructions (Gibco-BRL, Gaithersburg). When using the calcium phosphate method, cells were transfected as previously described (Bongarzone et al, 1993). Transformation foci were selected in DMEM with 5% calf serum. E25-427 cells, NIH3T3 cells overexpressing the human TRK proto-oncogene, were kindly provided by Dr. Mariano Barbacid and grown in DMEM supplemented with 10% calf serum. HEK 293T cells were grown in DMEM supplemented with 10% FCS and transiently transfected by calcium phosphate precipitation.

2.6 Cell stimulants and inhibitors

Cells were serum-starved for 12 or 24 hours prior to the addition of GDNF and EGF respectively. PP1 inhibitor [4-amino-5-(4-methylphenyl)-7-(-t-butyl)pyrazolo-D-3,4-pyrimidine] was obtained from BioMol Research Laboratories, dissolved in DMSO, and diluted into culture medium at the time of experimentation to a final concentration of 1 μ M. PP2, PP3, Genistein, and Herbimycin from Calbiochem (Novacalbiochem Corporation) were dissolved in DMSO and, diluted into the culture medium to final concentrations of PP2 (1 μ M), PP3 (1 μ M), Genistein (100 μ M), or Herbamycin (0.1 μ M).

Lactacystin was purchased from Sigma Chemical Co., dissolved in DMSO and diluted in culture medium to a final concentration

of 2 μ M. MG132 was purchased from Calbiochem (Novacalbiochem Corporation), dissolved in DMSO, and then diluted in culture medium to a final concentration of 10 μ M. Chloroquine, from Sigma Chemical Co., was dissolved in water and diluted in culture medium to a final concentration of 100 μ M. The human recombinant epidermal growth factor was from Life Technologies, Inc. (13247-051) and was diluted into the culture medium to a final concentration of 20 ng/ml.

2.7 Cell Motility Assay

Migration of RET-expressing cells was determined using an *in vitro* model of wound repair as described previously (Sato and Rifkin, 1988.). The semiconfluent monolayers were scraped with a 10 μ m sterile plastic micropipette tip, and cellular debris was removed by washing with serum-free DMEM. The monolayers were incubated in DMEM with or without 1 μ M PP1 for 12 h. Cell migration was observed using a phase contrast microscope under x200 magnification. Each condition was examined in duplicate, and the experiments repeated at least three times.

2.8 Immunofluorescent staining

Cells were seeded on chamberslides and immunostaining carried out after 24 hours to allow recovery of the cell surface protein portfolio after exposure to trypsin during harvesting. All

incubations were carried out at room temperature and antibodies were centrifuged prior to the addition to cells in order to remove any precipitate. Cells were washed 3 times in PBS pre-warmed to 37°C and fixed in 4% formaldehyde in PBS for 15 minutes. The fixative was removed by washing the chamber slides with PBS. Cells were incubated with BSA 5% in PBS for 30 minutes to reduce non specific binding of the secondary antibody. Cells were incubated in primary antibody diluted in PBS with or without 0.1% Triton X-100. Immunostaining with primary antibodies was followed by incubation with either rhodamine-conjugated antirabbit (Dako) or FITC-conjugated anti-IgG from Jackson ImmunoResearch (West Grove, PA). Confocal images were obtained using a Bio-Rad MRC-1024 confocal microscope.

2.9 Immunoprecipitation and Western Blot Analysis

Cells were washed with ice-cold PBS and lysed on ice for 20 minutes in modified radioimmune precipitation assay (RIPA) buffer (50 mM Hepes, pH7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), 1% sodium deoxycholate, 100 mM NaF, 0.5 mM Na₃VO₄, 1mM EGTA, 1% Nonidet P-40, 0.1 U/ml aprotinin, 1mM phenylmethylsulphonylfluoride (PMSF), 10 µg/ml leupeptin and 4 µg/m pepstatin A). Protein samples were immunoprecipitated with the specific antisera. Protein concentration was estimated

by a modified Bradford assay (Bio-Rad, Munich, Germany). Antibodies were added to lysates containing equal amounts of protein, and samples were incubated on ice for 1 hour. To precipitate antibody-antigen complexes, 40 μ l of a 50% suspension of Protein-A or protein-G sepharose beads (Amersham Pharmacia) were added to the lysate following rotation at 4°C for at least 2 hours. The immunoprecipitates were collected by centrifuging and washed three times in wash buffer (lysis buffer without detergents). Pellets were then boiled in SDS sample buffer and immune complexes were separated by electrophoresis on 8% SDS-PAGEs, transferred onto nitrocellulose membranes (Millipore Co., Bedford, MA), and analyzed by immunoblotting with the appropriate antibodies. For whole cell lysates, cells were lysed in SDS lysis buffer (62.5 mM Tris-HCl (pH 6.8) and 2% SDS). Total extracts were separated by SDS-PAGE.

Immunoreactive bands were visualized using horseradish peroxidase-conjugated antirabbit or antimouse antisera (Santa Cruz Biotechnology) and detected with the enhanced chemiluminescence kit (enhanced chemiluminescence detection reagents; Amersham Pharmacia Biotech, Little Chalfont, United Kingdom).

Antibodies used in this study were anti-RET polyclonal antibody (C-19) raised against a peptide corresponding to an amino acid sequence mapping at the COOH terminus of RET was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). To stain RET at the cell surface in experiments of immunofluorescence, we used a monoclonal non-purified serum directed against the extracellular domain, kindly provided by Dr. Tagliabue (Istituto Nazionale Tumori, Milano). The following antibodies were also used: mouse antiphosphotyrosine (4G10; Upstate Biotechnology, Inc., Lake Placid, NY); Vinculin monoclonal antibody (V-4505; Sigma Chemical Co., St. Louis, MO); Tubulin polyclonal antibody (T-3526; Sigma Chemical Co.); TRK polyclonal antibody (Santa Cruz Biotechnology); EGFR polyclonal antibody (sc-03; Santa Cruz Biotechnology, CA); ubiquitin monoclonal antibody (MMS-258; Babco) for Western blots; and ubiquitin monoclonal antibody (FK1, which recognizes only polyubiquitin-protein conjugates; Affiniti BioReagents, Inc.), Cbl polyclonal antibody (C-15 Santa Cruz Biotechnology); and clathrin monoclonal antibody (MA1-065, Affiniti BioReagents, Inc.), anti-Shc rabbit polyclonal antibody (UBI), anti-Crk monoclonal antibody (Transduction laboratories), anti-Ptyr (UBI), anti-Src (clone GD11) mouse monoclonal antibody (UBI), anti-Shp2 rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-p38 MAP kinase and anti-phosphop38 MAP kinase (Santa Cruz

Biotechnology). FITC-labeled phalloidin (Sigma Chemical Co.) was used to detect filamentous actin (final concentration 125 ng/ml).

Secondary antibodies were from Santa Cruz Biotechnology.

2.10 RET Kinase Activity

RET kinase activity was assessed *in vitro* by its ability to autophosphorylate and phosphorylate myelin basic protein (MBP; Sigma). Protein extracts were immunoprecipitated with anti-RET or anti-TRK antibodies as described above and washed twice with lysis buffer and once with incubation buffer (50 mM HEPES (pH 7.2), 20 mM MnCl₂, and 5 mM phenylmethylsulfonyl fluoride) and subjected to a kinase assay with MBP (Myelin Basic Protein) used as an exogenous substrate [-32P]ATP and PP1 (1 μ M) or PP2 (1 μ M), where indicated. After a 15-min incubation at 4°C in 20 μ l of the same buffer containing 4 μ Ci [-32P]ATP diluted with unlabeled ATP to a final concentration of 26 pmol ATP/sample and 50 μ M MBP, the reactions were stopped by the addition of reduced Laemmli buffer, and the products were heated at 100°C for 5 min. The radiolabeled RET and MBP proteins were resolved by SDS-PAGE and visualized by autoradiography. The intensity of the bands corresponding to autophosphorylated RET and phosphorylated MBP was quantified by PhosphorImager (Molecular Dynamics) analysis and expressed as the fold

increase relative to unstimulated wild-type RET. Control reactions in which RET immunoprecipitates were omitted showed no MBP phosphorylation (data not shown). Immunoprecipitation of equal amounts of RET was verified by anti-RET Western blotting and the quantity measured using ¹²⁵I-labeled protein A. The intensity of the bands corresponding to RET was quantified by PhosphorImager (Molecular Dynamics) analysis.

2.11 p13-suc1 capture and immunoblotting

The Frs2 protein was isolated from cell lysates by incubating equal amounts of protein with p13suc1-agarose (Oncogene Science) for 3 h at 4°C. P13suc1-agarose-captured proteins were washed three times with the above mentioned lysis buffer and boiled in Laemmli buffer for 5 min before electrophoresis. Captured proteins were subjected to 8% SDS-polyacrylamide gel, under reducing conditions, transferred to a nitrocellulose membrane (Millipore Co., Bedford, MA) and immunoblotted with an anti-Ptyr antibody (UBI). The identity of phosphorylated bands was further confirmed by subsequently re-probing with anti-RET and anti-Shp2 rabbit polyclonal antibodies (Santa Cruz Biotechnology).

2.12 Src kinase assay

To determine Src activation, c-Src was assayed for its ability to undergo autophosphorylation and phosphorylate a synthetic Cdc2 peptide, as follow: Src was immunoprecipitated with anti-Src (clone GD11 UBI) antibody from PLCLB-solubilised cell extracts as described (Borrello *et al.*, 1996). Src activity was assayed according to Cheng *et al.* (Cheng *et al.*, 1992) using the Src tyrosine kinase family-specific Cdc2 peptide substrate (Upstate Biotech-UBI). Immunoprecipitated anti-Src, prepared as described above, were incubated in 40µl of Src kinase reaction buffer (100mM Tris-HCl, pH 7.4, 0.2mM EGTA, 250mM Na₃VO₃, 40mM Mg(Ac) 10µl of cdc2 peptide (Upstate Biotech-UBI), 5 µl of cold ATP (25µM), and 2.5µCi of [γ -³²P]ATP. After 15 min of incubation at 30°C, reactions were terminated by the addition of 20 µl of 40% trichloroacetic acid and incubated for an additional 5 min at room temperature. Aliquots were subsequently blotted on to p81 phosphocellulose paper (Upstate Biotech-UBI). The p81 phosphocellulose paper was washed three times (5 min/wash) with 0.75% phosphoric acid and once with acetone at room temperature and the radiolabeled Src kinase substrate was counted in a liquid scintillation counter.

2.13 DNA extraction

2.13.1 From ES cells

The lysis buffer (100mM Tris Hcl pH 8.5, 0.5M EDTA, 10% SDS, 5M NaCl, 20mg/ml Proteinase K) is added directly to the cells. Lysis of the cells was carried out at 37°C overnight with agitation.

To precipitate the DNA; one volume of isopropanol was added to the lysate and the samples were mixed or swirled until precipitation was complete (about 10-20 min).

The DNA was then recovered by lifting the aggregated precipitate from the solution using a disposable yellow tip. Excess liquid was dabbed off and the DNA was placed in a pre-labelled Eppendorf tube containing, depending on the size of the precipitate, 20 to 500ul to 10mM Tris HCl, 0.1mM EDTA, pH 7.5.

2.13.2 DNA from Tail Biopsies

Tails were removed into polypropylene microfuge tube and 0.5 ml DNA digestion buffer (50mM Tris-HCl pH 8.0, 100mM EDTA pH 8.0, 100mM NaCl, 1% SDS, proteinase K 0.5mg/ml) added prior to incubation overnight at 50-55°C with gentle shaking.

To extract DNA, 0.7ml neutralized phenol/chloroform/isoamyl alcohol (25:24:1) was added and spun in a microcentrifuge at top speed for 5 minutes before transferring 0.5 ml of the upper phase to a new microcentrifuge tube.

1 ml 100% ethanol at room temperature was added and inverted (using a clinical rotator) until a DNA precipitate formed

(approximately 1 minute). The tube content was then spun in a microcentrifuge for 5 minutes and the supernatant was removed and discarded carefully. 0.5-1ml 70% ethanol (-20°C) was then added and inverted several times before spinning in microcentrifuge for 5 minutes in order to carefully remove and discard the supernatant.

The tubes were then spun to remove the last drops of ethanol solution with a 25µl capillary tube. The pellet was air dried at room temperature and then 100-200µl TE buffer was added before incubation at 65 °C for 15 minutes to resuspend DNA.

2.14 Southern blot analysis

For Southern, 5-10 µg of genomic DNA was digested with the appropriate enzyme and run on 0.7% agarose gels in 0.5X TBE. DNA was transferred to a nylon membrane (Hybond-N, Amersham Int., UK) and subsequently hybridized with an [α -³²P]dATP random-primed-labelled probe consisting of a PCR fragment from the RET coding region. Hybridization was done in 6X SSC (1X SSC is 0.15 M NaCl, 15 mM Sodium-Citrate, pH 7.0), 0.1% SDS, 250 mg/ml herring sperm DNA and 50% formamide, at 42°C overnight. After hybridization, membranes were washed with:

1X, 15 minutes, room temperature, 2X SSPE/0.1% SDS

1X, 30 minutes, room temperature, 0.1X SSPE/0.1% SDS

1X, 30 minutes, 37°, 0.1X SSPE/0.1% SDS

2X, 30 minutes, 68°, 0.1X SSPE/0.1% SDS

Membranes were then exposed to a autoradiographic film for 96 hr.

3. FUNCTIONAL ANALYSIS OF CYS MUTANTS ASSOCIATED BOTH WITH MEN2A AND HSCR DISEASE

3.1 Introduction

In rare families, HSCR and MEN2A or FMTC can co-occur because of the same RET mutation (Attie et al, 1995; Mulligan et al, 1994). These combined phenotypes are generally associated with a cysteine to arginine substitution in exon 10 of RET, especially at position 609, 618 and 620. Recent evidence has established that the great majority of these mutated RET proteins translocate to the cell surface with low efficiency (Ito et al, 1997; Cariomagno et al, 1997; Chappius-Flament et al, 1998; Takahashi et al, 1999) and therefore phosphorylated RET dimers accumulate within intracellular endosomal compartments (Ito et al, 1997; Carlomagno et al, 1997; Chappius-Flament et al, 1998; Takahashi et al, 1999 and our unpublished results). As a consequence, an insufficient amount of the mature RET receptor is available; a deficiency which compromises the correct development of the enteric nervous system. On the other hand, this aberrant distribution of the cysteine mutated RET receptor is still constitutively activated, and does not impair mitogenic signalling in the thyroid/adrenal glands, thus leading to hyperplasia or tumour formation.

Dimerization of the RET receptor, either due to the presence of cysteine mutations or to the binding of its ligand, results in autophosphorylation of intracellular tyrosine residues and creation of docking sites for a number of SH2-containing signal transduction molecules. These include GRB7 and GRB10, phospholipase C γ (PLC γ), SHC, GRB2, Enigma and FRS2 (Arighi et al, 1997; Alberti et al, 1998; Soler et al, 1999; Asai et al, 1995) . Phosphatidyl 3'-kinase (PI3K) (Soler et al, 1999; Segouffin-Cariou and Billaud, 2000) has also been implicated in RET-mediated cell proliferation, survival and motility (Marte and Downward, 1997). Recent works have reported that Src activity is necessary for the neuronal survival mediated by GDNF family ligands mediated (Encinas et al, 2001) and is required for the recruitment of RET to lipid rafts (Paratcha et al, 2001). It is well accepted that proteins can trigger different signalling according to their cellular compartmentalisation. I here have attempted to dissect the signaling pathways driven by a mislocalised HSCR/MEN2A associated mutation (RET^{C620R}) from the ones driven by the typical MEN2A associated mutation (RET^{C634R}) correctly expressed at the cell surface. This was done in order to establish the key events in RET mediated signaling that predispose for HRSC disease in patients carrying the C620R substitution.

3.2 Analysis of RETC620R and RETC634R localisation by confocal microscopy

To compare the functional consequences of mutations affecting the cysteine rich domain of RET, we introduced individual mutations by substituting cysteines 620 and 634 for an arginine in the cDNA coding for the short RET isoform. Lysates prepared from NIH3T3 cells stably expressing the RET cysteine mutants were immunoprecipitated with an anti-RET serum and the resulting immunocomplexes were analysed by SDS-polyacrylamide gel electrophoresis. Western blotting of these samples with an anti-RET polyclonal antibody revealed two products of 140 kDa and 160 kDa, in RET^{C634R} whereas in the RET^{C620R} mutant, the 160 kDa product was almost undetectable. Probing the same filter with an anti-phosphotyrosine monoclonal antibody revealed that the 140 kDa product, corresponding to the partially glycosylated form of RET was phosphorylated in RET^{C620R} mutants whereas in RET^{C634R} mutants, the most phosphorylated band corresponded to the 160 kDa product (Fig 1). These results confirmed previous data which suggested that the Cys-620 mutation results in a decrease of the level of RET at the cell surface. (Ito et al, 1997; Carlomagno et al, 1997; Chappius-Flament et al, 1998; Takahashi et al, 1999 and our unpublished results).

To further verify these findings, NIH3T3 cells expressing RET^{C620R} or RET^{C634R} short isoform, were also examined by immunofluorescence confocal microscopy using two different anti-RET polyclonal antibodies, one specific for the carboxy-terminal of RET and the other for the extracellular domain. The carboxy-terminal specific antibody detected the presence of both mutant receptors within intracellular compartments (Fig 1, green staining). Signal was particularly intense in perinuclear regions, suggesting a prevailing localisation in the endoplasmic reticulum. Furthermore, the C634R mutant receptor but not C620R, was localised at the cell surface, as revealed by surface staining obtained with the extra cellular specific antibody in Fig 1, red staining.

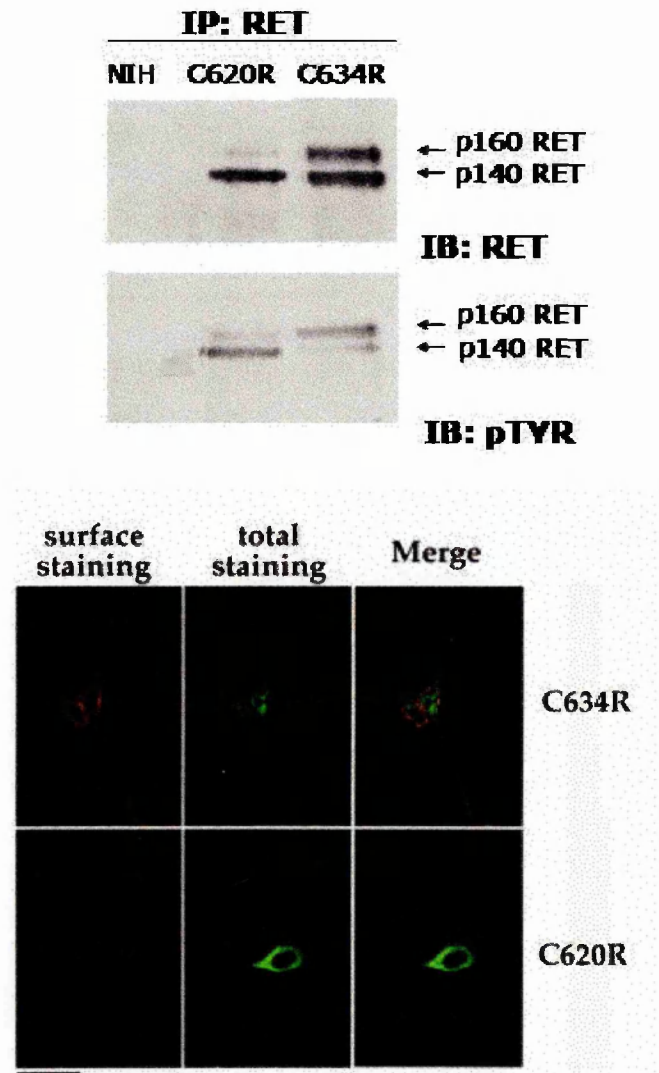


Fig.1

Fig. 1 RET^{C634R} mutant receptor, but not RET^{C620R} is localised at the cell surface. Cell lysates from untransfected and RET^{C634R} and RET^{C620R} transfected NIH3T3 cells were immunoprecipitated with anti-RET, subjected to SDS-PAGE and subsequently blotted with anti-RET and anti-Ptyr antibodies. The clones were also examined by

immunofluorescence confocal microscopy. For surface localisation, cells were incubated at 4°C with anti-RET extracellular specific antibody. They were then fixed, permeabilised and double stained with the carboxy-terminal specific antibody to reveal the intracellular distribution of the receptor. Note that RET^{C634R} mutant receptor but not RET^{C620R}, is localised at the cell surface, as revealed by surface staining obtained with the extracellular specific antibody (red staining). Staining with the carboxy-terminus specific antibody detects the presence of both mutant receptors within intracellular compartments (green staining). Bar: 50 µm.

3.3 Different morphological features of RET^{C620R} and RET^{C634R} transfected cells

RET^{C620R} expressing cells examined by phase-contrast microscopy showed a phenotype similar to that of NIH3T3 fibroblasts, whereas RET^{C634R} expressing cells exhibited a bright, rounded morphology with very elongated processes. To better characterise the cytoskeletal changes associated to the expression of RET^{C620R} and RET^{C634R}, we performed immunofluorescence analysis with FITC-labelled phalloidin and vinculin antibodies in order to respectively examine filamentous actin (F-actin) and focal contacts. Cells were double stained with anti-RET and phalloidin or anti-RET

and anti-vinculin. RET^{C620R} positive cells, and to a higher extent RET-negative NIH3T3 cells (asterisks in fig 2A), showed a typical elongated fibroblastic shape and well organised actin stress fibers parallel to the long axis of the cell and spread throughout the cell body (Fig. 2A). The pattern of vinculin staining indicated a peripheral positivity reminiscent of focal adhesion complexes (Fig. 2B), as if RET^{C620R} expression in NIH3T3 fibroblasts could scarcely affect their morphology. In contrast, RET^{C634R} positive cells had characteristic features of transformed cells. They did not possess well-defined F-actin stress fibers, but rather presented cortical bundles of actin filaments visible as linear lines along the margin of the cells (Fig. 2A). Vinculin staining of RET^{C634R} was localised to the perinuclear regions and almost absent in the cell periphery, where a significant reduction in focal contacts was observed (Fig. 2B). These results highlighted important structural differences between RET^{C620R} and RET^{C634R} expressing cells that may also affect cell motility.

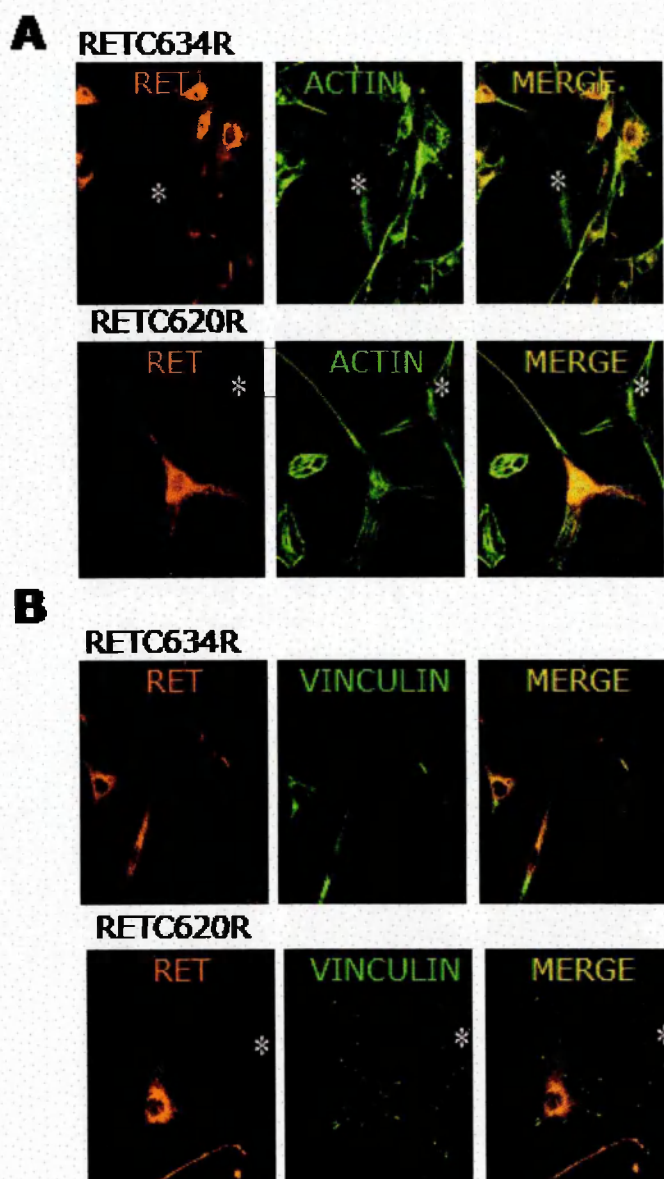


Fig.2

Fig. 2 RET^{C634R} expression in NIH3T3 cells induces changes in cell architecture. **(A)** RET and actin distribution in RET^{C634R} and RET^{C620R} expressing cells. To analyse stress fibers and actin organization, transfected cells were double stained with anti-RET

(red) and FITC-conjugated Phalloidin (actin, green). **(B)** *RET* and vinculin distribution in *RET*^{C634R} and *RET*^{C620R} expressing cells. In order to analyse focal contacts, transfected cells were double stained with anti-RET (red) and anti-vinculin (green) antibodies. The images were analysed by confocal microscopy. The asterisks indicate untransfected NIH3T3 cells not expressing the RET mutants. Bar: 50 μ m.

3.4 Different mobility of *RET*^{C620R} and *RET*^{C634R} expressing cells

To assess differences in the motility between the two cell types, an in vitro wound repair model (Sato et al, 1988) was used. Cells including the parental line were wounded, cell culture medium removed and the monolayers incubated. After 15 h from wounding, NIH3T3 untransfected cells and to a lesser extent *RET*^{C620R} expressing cells, showed no significant migratory potential (Fig. 2C). In contrast, C634R expressing cells demonstrated extensive migration into the denuded area (Fig. 2C).

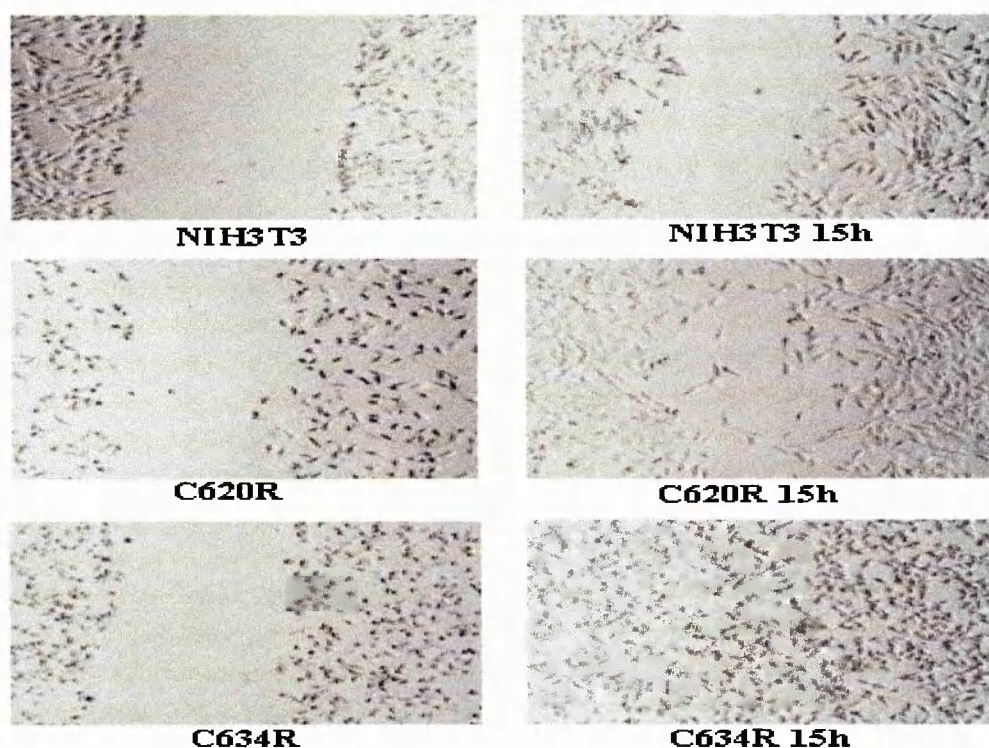


Fig2 C

Fig. 2 RET^{C634R} expression in NIH3T3 cells induces changes in cell architecture. **(C)** RET^{C634R} expressing cells show a consistent motile potential. Monolayers of untransfected NIH3T3 cells, RET^{C634R} and RET^{C620R} transfected cells were wounded and cultured in presence of serum for 15 hours. Cell migration into the denuded area was evaluated at 0 and 15 hours with a light microscope equipped with phase-contrast optics (original magnification 200X).

3.5 RET^{C634R} and RET^{C620R} recruitment of signaling molecules

Focus forming assays on NIH3T3 fibroblasts demonstrated that RET^{C620R} displayed a transforming activity although lower than the activity displayed by RET^{C634R} (Fig 3 upper panel). It was then tempting to speculate that both the p140 precursor and p160 mature forms of RET, when phosphorylated and dimerized, could transduce mitogenic signalling in spite of their different localization: the ER for RET^{C620R} and both the ER and the surface membrane for RET^{C634R}. Immunoblot analysis with anti-RET specific antibodies, confirmed that RET^{C620R} transfected clones almost exclusively expressed the p140 band, corresponding to the partially glycosylated form of RET. In order to explain the transforming activity of RET^{C620R}, we investigated its ability to transduce mitogenic signals. In particular, we studied whether both RET carrying the C620R and C634R mutation were able to bind to the Shc adapter protein and PLC γ as previously demonstrated for RET^{C634R} (Carlomagno *et al.*, 1997; Chappuis-Flament *et al.*, 1998). Cell lysates from RET^{C620R} and RET^{C634R} transfected NIH3T3 cells were immunoprecipitated with anti-Shc or anti-RET antibodies and subsequently blotted with anti-Ptyr antibodies. Both the precursor and mature forms of RET were co-immunoprecipitated with Shc phosphorylated proteins as already demonstrated for other RET mutants by Carlomagno *et al.* (Carlomagno *et al.*, 1997) (Fig. 3

lower panel). The possibility that RET mutant precursors interacted with Shc in intracellular endosomal compartments is supported by the presence of Shc proteins in the plasma membrane and endocytic structures as previously shown by others (Lotti et al, 1996).

Analogously, immunoprecipitations were carried out using anti-PLC γ and anti-RET antibodies and then blotted with anti-Ptyr or anti-RET antibodies. In RET^{C634R} transfected cells, tyrosine phosphorylated PLC γ was predominantly associated with the p160 form of RET. In RET^{C620R} transfected cells, most of the phosphorylated PLC γ protein was recovered associated with the p140 partially glycosylated form of RET (data not shown).

Therefore, intracellularly retained RET^{C620R} proteins strongly bind phosphorylated Shc and PLC γ , thus confirming their ability to induce mitogenic pathways driven by these two proteins independent of their cellular localization.

It has been recently demonstrated that RET activated by GDNF or by classical MEN 2A mutations (such as C634Y or C634R) interacts with Frs2 and directly complexes with Shp2 tyrosine phosphatases (Califano et al, 2000; Kurokawa et al, 2001). Frs2 has been shown to be involved in neuronal signaling, in particular it regulates cell cycle arrest and neuronal differentiation (Rabin et al, 1993). Interestingly, as for the TRKA receptor, (Meakin et al,

1999), the Frs2 binding site on RET, to tyrosine 1062, is concomitantly the binding site for Shc (Kurokawa et al, 2001) and it has also been demonstrated that competition between SHC and FRS2 at the Tyr-1062 binding site, can in part be involved in the switch between cell cycle progression/mitogenesis and cell cycle arrest/differentiation .

We therefore investigated whether the RET^{C634R} and RET^{C620R} isoforms interacted with the Frs2 signal transducer and Shp2. Cell lysates from RET^{C634R} and RET^{C620R} transfected cells were subjected to immunoprecipitation using agarose conjugated with the p13-suc protein, which specifically binds Frs2 and indirectly Shp2. The complex has been examined in immunoblot analysis using anti-Ptyr, and then serially reprobed with anti-RET, anti-Frs2 and anti-Shp2 antibodies (Fig. 3 lower panel). In immunoblot experiments, coimmunoprecipitation of all the isoforms of RET^{C634R} and RET^{C620R} with Frs2 and Shp2 phosphoproteins was detected. Therefore, the partially glycosylated form of RET carrying the C620R mutation, as well as RET with the C634R mutation is able to interact with Frs2. This supports the possibility that the intracellular membrane compartment can also transduce RET-Frs2 signaling.

Various data support the idea that despite the lack of an enzymatic kinase domain, Crk plays a crucial role in growth factor-stimulated signal transduction and regulation of the actin

cytoskeleton (Ishiki et al, 1997; Nakashima et al, 1999). Tyrosine-phosphorylated proteins known to be associated with actin stress fibers in adhesion foci, such as p130Cas or Paxillin, have been shown to interact with the SH2 domain of Crk. We investigated whether RET^{C634R} and RET^{C620R} recruited Crk in the respective transformed cells, which exhibited differences in the organisation of their focal adhesions (Fig 2). Phosphorylated RET^{C634R} and RET^{C620R} were detected in Crk immunoprecipitates from both transfected cell types (Fig 3 lower panel). Therefore, since Crk adapter protein can bind and possibly trigger specific signaling from both RET mutants, the observed differences in focal adhesion complexes found in the transformed cells, could suggest the existence of distinct Crk roles, possibly involved in different signalling pathways and cellular processes.

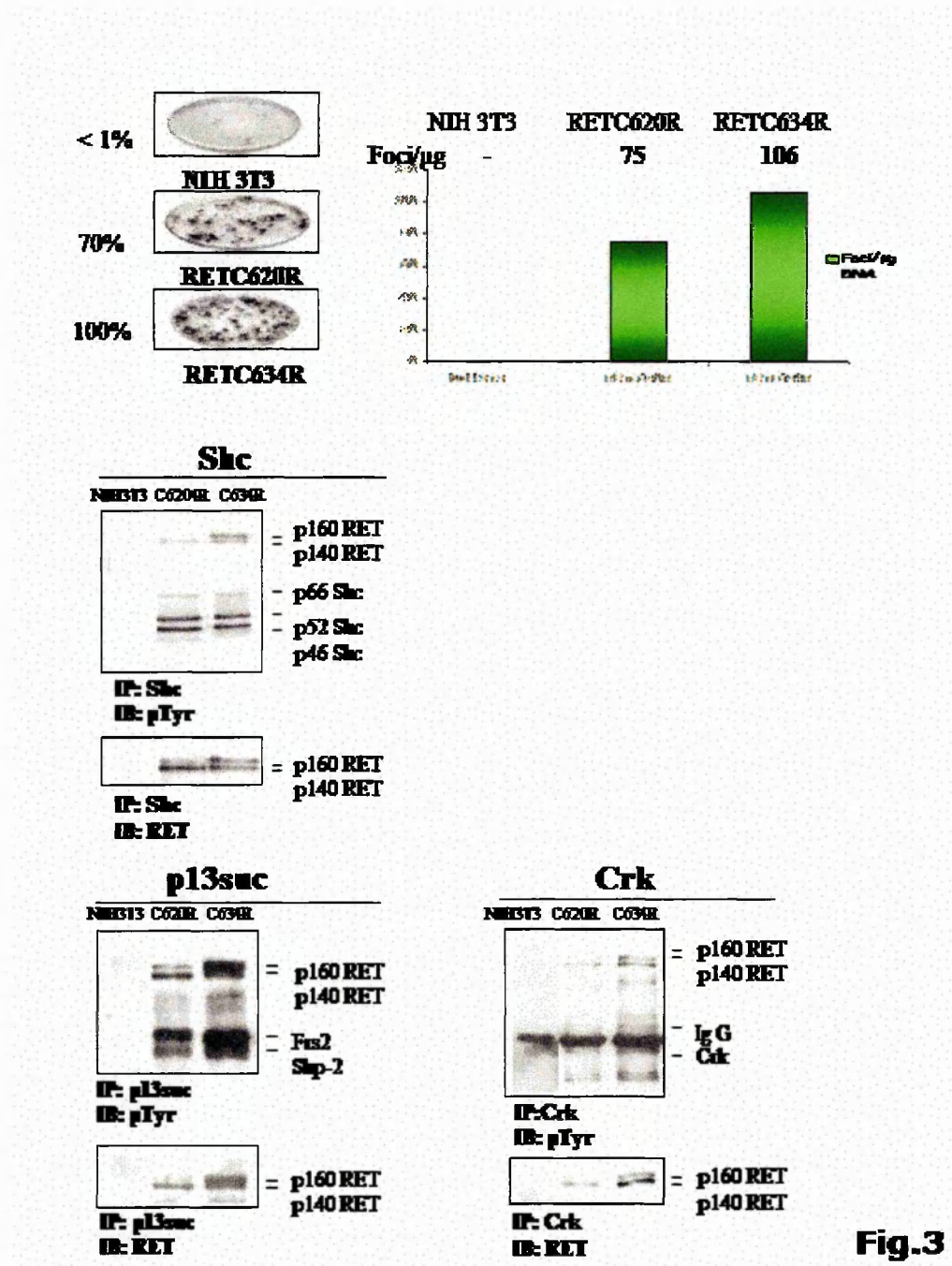


Fig.3

Fig. 3 *RET^{C634R} and RET^{C620R} recruitment of signaling molecules.* RET transforming activity was assayed transfecting NIH3T3 cells with *RET^{C634R}* and *RET^{C620R}* and the number of foci counted. The

results are of four independent experiments, and expressed as the number of foci per micrograms of transfected DNA (Upper panels). Cell lysates from untransfected and RET^{C634R} and RET^{C620R} transfected NIH3T3 cells were immunoprecipitated with anti-Shc or anti-RET subjected to SDS-PAGE and subsequently blotted with anti-Ptyr antibodies. Cell lysates were also subjected to immunoprecipitation using both anti-RET and agarose conjugated with the p13-suc protein, which specifically binds Frs2 and indirectly Shp2. p13suc immunoprecomplexes were separated on SDS-PAGE, analysed in Western blotting with anti-Ptyr antibodies and then serially re-probed with anti-RET, anti-Frs2 and anti-Shp2 antibodies. Cells lysates were also subjected to immunoprecipitation with anti-Crk antibodies, subjected to SDS-PAGE and phosphorylated bands visualised with anti-Ptyr antibodies. RET immunoprecipitates were analysed to asses RET protein phosphorylation in each cell type (lower panels).

3.6 High Src activity in RETMEN 2A expressing cells

Since Src has been implicated in RET mediated mitogenic activity and is found to co-immunoprecipitate *in vivo* with activated RET (Melillo et al, 1999), we evaluated whether the mutants, RET^{C634R} and RET^{C620R}, were able to interact with Src. We immunoprecipitated Src kinase using specific antibodies and analysed the presence of RET mutants using RET specific antibodies. Both the mutants were present in Src

immunoprecipitates (data not shown). Since the Src protein can be phosphorylated on tyrosine residues in an inactive or active state (Cooper and Howell, 1993; Nada et al, 1991) we used an *in vitro* kinase assay to establish the effects of C634R and C620R on Src activity. Src activity was measured by the capacity of Src immunoprecipitates from untransfected NIH3T3, RET^{C634R} and RET^{C620R} expressing cells to tyrosine phosphorylate the synthetic Src specific cdc2 peptide (Church et al, 1994). RETC634R expressing cells showed a pronounced kinase activity, 3 to 4-fold higher than the one found in RET^{C620R} cells, whose level correlated with that of normal parental NIH3T3 cells (Fig. 4).

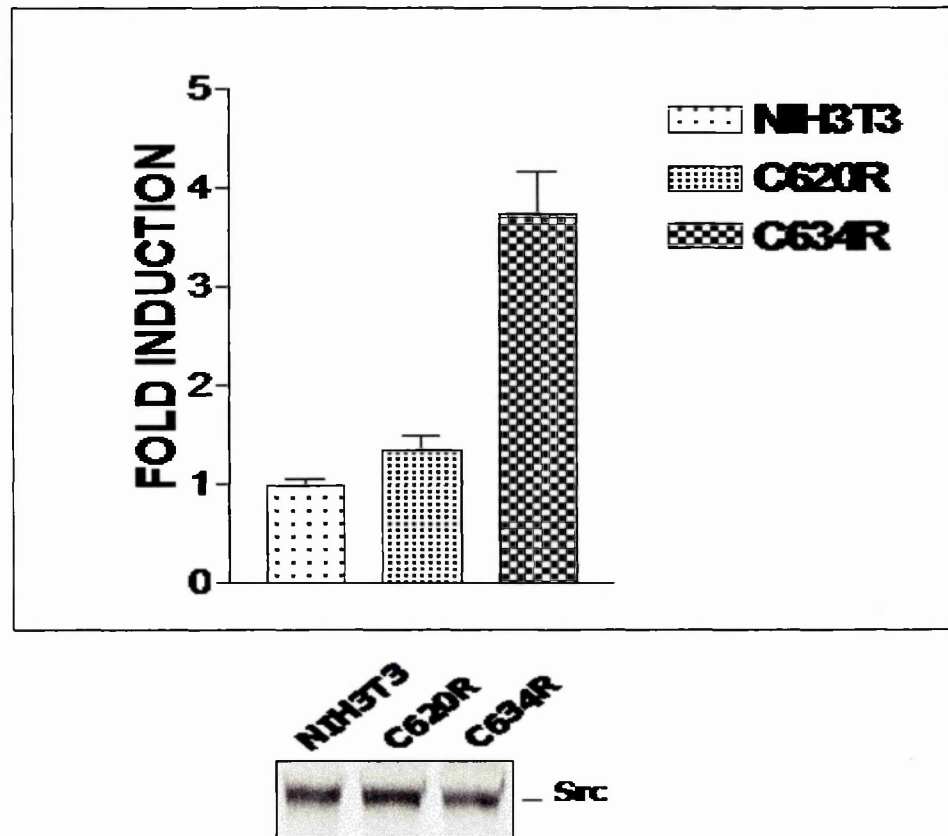


Fig.4

Fig. 4 *High Src activity in RETC634R expressing cells.* Cell lysates were prepared from serum starved cells and were immunoprecipitated with anti-Src antibodies. Src immunoprecipitates were subjected to an *in vitro* kinase assay using the the synthetic Src specific cdc2 peptide as a substrate. Kinase activity was evaluated by counting the cpm incorporated by radiolabelled cdc2. The results of four independent experiments are expressed as fold of induction with respect to untransfected NIH3T3 cells. Data represent the means \pm SD of the values. (upper

panel). Src immunoprecipitates were analysed by Western blotting with an anti-Src antibody to control protein expression in untransfected NIH3T3 cells, RET^{C634R} and RET^{C204R} transfected cells (bottom panel).

Since RET is physiologically expressed in cells of neuroectodermic origin, the human neuroectodermic SK-N-MC cell line represents a model in which to study RET signaling. The SK-N-MC cell line was stably transfected with RET wild type, RET^{C634R}, RET^{C620R}, RET^{C609W} and RET^{S765P} and RET expression and phosphorylation checked. We then investigated whether, in this more physiological context, differences in activating Src were detected between the RET mutants, as observed in NIH3T3 cells.

Src activity is regulated by tyrosine phosphorylation at two sites with opposing effects. Phosphorylation of Tyr416 in the activation loop of the kinase domain upregulates the enzyme, whereas phosphorylation of Tyr527 renders the enzyme less active (Hunter, 1987; Nada et al, 1991). An antibody that detects Src only when activated by phosphorylation at Tyr416 was used in Western blot experiments. It was demonstrated that Src phosphorylation was present only when proteins were extracted from RET^{C634R} transfected cells (data not shown). In fact, as

observed in NIH3T3 cells expressing the different Cysteine RET mutants, Src seemed to be inactive in RET^{C620R} transfected SK-N-MC cells. Moreover, the Tyr416 phosphorylation was also absent when Src phosphorylation was assessed in the RET^{C609W} and RET^{S765A} mutants, thus again suggesting that Src activity might indeed play a crucial role, which is probably impaired in Hirschsprung disease.

3.7 High p38 MAP kinase phosphorylation in RETMEN 2A expressing cells

Previous studies have demonstrated that RET stimulates Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) signaling (Chiariello et al, 1998). We analysed the kinase activity of p44/p42, JNK and p38 MAPKs. We did not find significant differences in the activation level of ERK1, ERK2 and JNK in RET^{C634R} and RET^{C620R} expressing cells but a higher phosphorylation of p38 in RET^{C634R} cells was clearly detected (Fig. 5). This activity could be related to cytoskeletal rearrangements and/or the motility we found in RET^{C634R} expressing cells. In fact, the p38 pathway is involved in cell migration and organisation of the actin cytoskeleton (Matsumoto et al, 1999). This could be achieved in virtue of ability that p38 has in controlling and

coordinating cellular activities such as gene transcription and adhesion (Hall et al, 1998). Moreover, consistent with our data, the expression of the v-Src oncoprotein has been shown to strongly stimulate p38-MAPK in different cell types (Turkson et al, 1999; Lee et al, 1999). However, little is still known about the contribution of Src family kinases to the signaling pathways which link RET to p38-MAP activation.

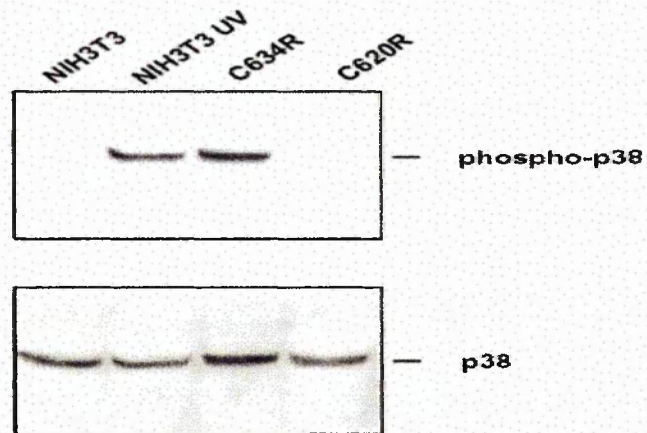


Fig. 5

Fig. 5 High p38 MAP kinase phosphorylation in RETC634R expressing cells. Total cell lysates from transfected and untransfected NIH3T3 cells, serum starved for 20 hours, were subjected to SDS-

PAGE and immunoblotted with anti-p38 (top) and anti-phospho p38 antibodies (bottom). Where indicated, NIH3T3 cells after starvation were treated with UV irradiation. Similar results were obtained performing three independent experiments.

3.8 GDNF induces scattering of SK-N-MC neuroepithelioma cells expressing RET^{C634R} but not SK-N-MC cells expressing Hirschsprung associated RET mutations.

In order to test the effects of the expression of RET Cys mutations in a more physiological context, since RET is expressed in cells of neuroectodermal origin, the human neuroectodermal SK-N-MC cell line was stably transfected with RET wild type, C634R, C620R, C609R, and S765P and RET expression and phosphorylation was checked.

Ligand-induced activation of RET in SK-N-MC neuroepithelioma cells stably transfected to express RET wild type, generates a "scattering" response, as demonstrated by van Puijenbroek (van Puijenbroek *et al*, 1997). In parental SK-N-MC cells, scattering is also induced by treatment with bFGF (van Puijenbroek *et al*, 1997). Cell scattering is characterised by several morphological changes such as the loss of epithelial features and cell dissociation with consequent individual movement. In order to determine the ability of RET cysteine mutants to respond to GDNF, we took advantage of this

system. SK-N-MC cells stably transfected to express RET wild type, RET^{C634R}, RET^{C620R}, RET^{C609W} and RET^{S765P} were therefore cultured and stimulated with GDNF. GDNF induced-scattering was clearly visible for those clones expressing RET wild type and RET^{C634R} but not for all the other RET mutants, RET^{C620R}, RET^{C609W} and RET^{S765P} (Fig 6), that are associated with Hirschsprung's disease. The scattering effect was associated with an increase in RET phosphorylation, assessed when RET was immunoprecipitated and its tyrosine phosphorylation checked by anti-Ptyr Western blotting. These results clearly demonstrated that GDNF induced a considerable enhancement of RET^{C634R} autophosphorylation together with a scattering effect, despite its constitutive activation. On the contrary all RET mutants associated with Hirschsprung's disease did not respond to GDNF since neither an increase in the levels of RET autophosphorylation nor a scattering effect was detectable. This is an interesting result since it has been demonstrated that GDNF promotes the migration of enteric neural crest-derived cells and may be important for driving neural crest cell migration along the gut, preventing them from straying within the mesentery and in promoting axon outgrowth (Young et al, 2001). Consistent with this, the RET mutants associated with Hirschsprung's disorders analysed were unable to respond to GDNF treatment, which should result in cell dissociation

and consequent individual movement.

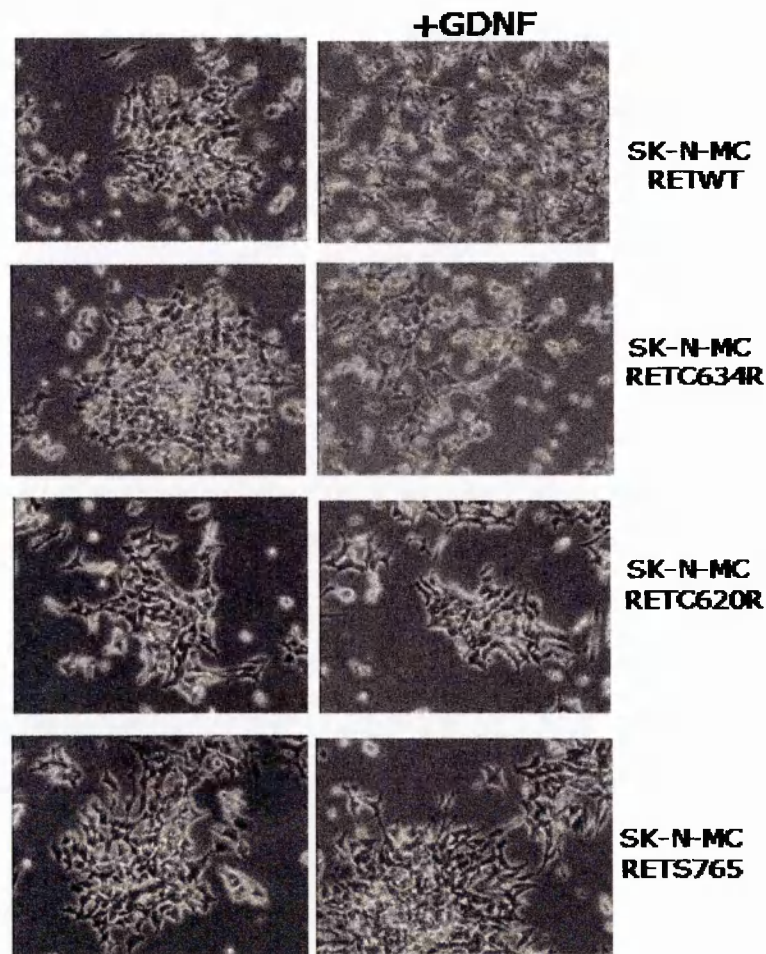


Fig.6

Fig. 6 GDNF induces scattering of SK-N-MC neuroepithelioma cells expressing RET^{C634R} but not of SK-N-MC cells expressing Hirschsprung associated RET mutations. SK-N-MC cells stably transfected to express RET wild type, RET^{C634R} , RET^{C620R} and RET^{S765}

were cultured after starvation in the presence or absence of 50nM GDNF and, the scattering effect observed.

3.9 Discussion

As a paradox, some cysteine mutations of RET such as the RET Cys⁶²⁰ substitution are oncogenic in neuroendocrine cells and responsible for MEN2A and FMTC, but at the same time, they can impair enteric neuronal development leading to HSCR disease. In order to address the question of why certain substitutions predispose to HSCR disease, we compared the biological and biochemical features of cells transfected with RET carrying either the Cys⁶²⁰ or the Cys⁶³⁴ mutation that is commonly found in MEN2A and FMTC patients, but does not affect neuronal development.

In agreement with previously reported biochemical results, our confocal microscopy analysis of RET localisation demonstrated that RET^{C620R} primarily accumulates in intracellular endosomal compartments and is almost absent at the cell surface. In contrast, RET^{C634R} proteins appeared to be localised at the cell surface as well as intracellularly. Moreover, although differently localised within cells, the RET^{C620R} and the RET^{C634R} mutants activate similar mitogenic pathways but display characteristic morphological features. RET^{C634R} expression drives cellular modifications, including

cell rounding and increased motility due to adhesion loss and disruption of the actin cytoskeleton. These changes are not associated with RET^{C620R} transformation of NIH3T3 cells. The expression pattern of RET^{C620R} and the associated morphological features are accompanied by low levels of Src kinase activity, whereas RET^{C634R} drives a sustained activation of this signalling molecule.

As the induction of RET-mediated Src activity requires RET localisation to lipid rafts (Tansey *et al*, 2000), our results support the idea that RET mediated activation of Src signaling pathways is less efficient in those mutants retained within the ER. Although no data on any RET variant localisation in lipid rafts has been reported yet, our findings are in line with previously reported data demonstrating that cellular Src as well as v-Src, was associated with perinuclear endosomal membranes in its inactive state (David-Pfeuty and Nouvian-Dooghe, 1990) and upon activation, it complexed with actin and translocated to focal adhesions at the cell periphery.

From a functional point of view, it is well accepted that Src is a co-ordinator of specific events that control cellular morphology that give rise to an altered growth pattern, cell rounding and increased motility as a consequence of adhesion loss and disruption

of the actin cytoskeleton, as clearly demonstrated both for transformed fibroblasts and neuronal cells. Expression of activated Src in fibroblasts alters cell morphology and reduces cell adhesion to the extracellular matrix (Brown *et al*, 1996). Src associates directly with the Focal Adhesion Kinase (FAK) and localises to focal complexes where it stimulates focal contact turnover (Fincham *et al*, 2000). The consequent loosening of cellular adhesion strength allows cells to easily detach and move (Fincham *et al*, 2000). Cultured neurones lacking either Src or Fyn exhibit defects in neurite outgrowth in response to cell adhesion molecules and mice lacking Src and Fyn kinases exhibit defects in axon guidance and fasciculation (Beggs *et al*, 1994); specifically, Src activity results concentrated in growth cones, where it affects the regulation of actin dynamics in response to extracellular signals (Lipfert *et al*, 1992).

The RET^{C620R} variant, when expressed in the neuroectodermic SK-N-MC cell line, together with the inability to induce Src activity, also failed to respond to GDNF stimulation. Addition of GDNF neither increased RET^{C620R} and RET^{C609R} phosphorylation levels nor induce cell scattering, since their intracellular location impeded their interaction with the membrane associated GDNF/coreceptor complex. GDNF promotes the migration of enteric neural crest-derived cells and it is important for driving neural crest cell

migration along the gut, in preventing them from straying within the mesentery and in promoting axon outgrowth (Young et al, 2001).

In conclusion, here we propose a model in which the mislocalised RET^{C620R} mutant, although able to trigger mitogenic signaling even if retained within intracellular compartments, fails in activating Src and responding to GDNF. This thus compromises cellular morphological modifications essential for neuroenteric precursor development and movement, leading to HSRC disease.

4. PP1 INHIBITOR INDUCES DEGRADATION OF RETMEN2A AND RETMEN2B ONCOPROTEINS THROUGH PROTEOSOMAL TARGETING

4.1 Introduction

The study of the activity of Src in RET transformed cells has been hampered by the lack of specific inhibitors. PP1, which is thought to be a Src-family-selective small inhibitor, was shown to have interesting effects on RET expressing cells. The knowledge of the availability of inhibitors specific for RET oncoproteins could provide new tools capable of highlighting the physiologically and pathologically activated pathways involved and help in developing new therapeutic strategies for RET-associated diseases. The inhibition of tyrosine kinase activity by small, cell-permeable molecules is a promising approach to target oncoproteins and has already reached clinical application for ErbB/HER subgroup of receptors (Mendelsohn et al, 2000; Dancey et al, 2001). Carlomagno et al. have very recently demonstrated that the pyrazolo-pyrimidine PP1 blocks tumorigenesis induced by RET/PTC cytoplasmic oncogenes by inhibiting RET enzymatic activity and its transforming effects (Carlomagno et al, 2002). We have further explored the mechanism of action of the inhibitor, specifically showing that PP1 abrogates the cellular morphological

modifications induced by the expression of different cytoplasmic or membrane-bound RET oncoproteins in NIH3T3 cells, restoring the characteristic fibroblast actin organization and focal adhesion formation. We also present evidence that, along with the inhibition of tyrosine phosphorylation, PP1 induces proteosomal destruction of activated receptors. In fact, in the presence of the inhibitor the receptors are rapidly targeted to the degradative pathway and the intracellular destruction of ubiquitinated RET oncoproteins results accelerated. Taken together, our results suggest that PP1 treatment may represent a promising new strategy to selectively target RET oncogenic products for destruction and could be used to develop new therapeutic techniques for medullary thyroid cancer therapy, which presently responds very poorly to chemotherapeutic agents.

4.2 PP1 reverts the transformed morphology and affects motility of cells expressing RET/MEN2 oncoproteins.

In agreement with what has been already described by Carlomagno et al. (Carlomagno et al, 2002), PP1 evidently effects the morphology of fibroblasts expressing RET carrying the C634R and M918T substitutions, which are the most important MEN2A

and MEN2B associated mutations. These cells, examined by phase-contrast microscopy (Fig 1A), exhibited a bright, rounded morphology with elongated processes very different from the typical phenotype of parental NIH3T3 cells. In the presence of PP1 at 1 μ M for 12 hours, they appear to revert their morphology, becoming similar to untransformed NIH3T3 fibroblasts. As expected, PP1 exerted also clear effects on RET/PTC3 cell morphology as well. As control, parental NIH3T3 cells and NIH3T3 expressing TRKA were also examined by phase-contrast microscopy, yet they did not show any significant change in their morphology upon PP1 treatment (Fig. 1A).

A

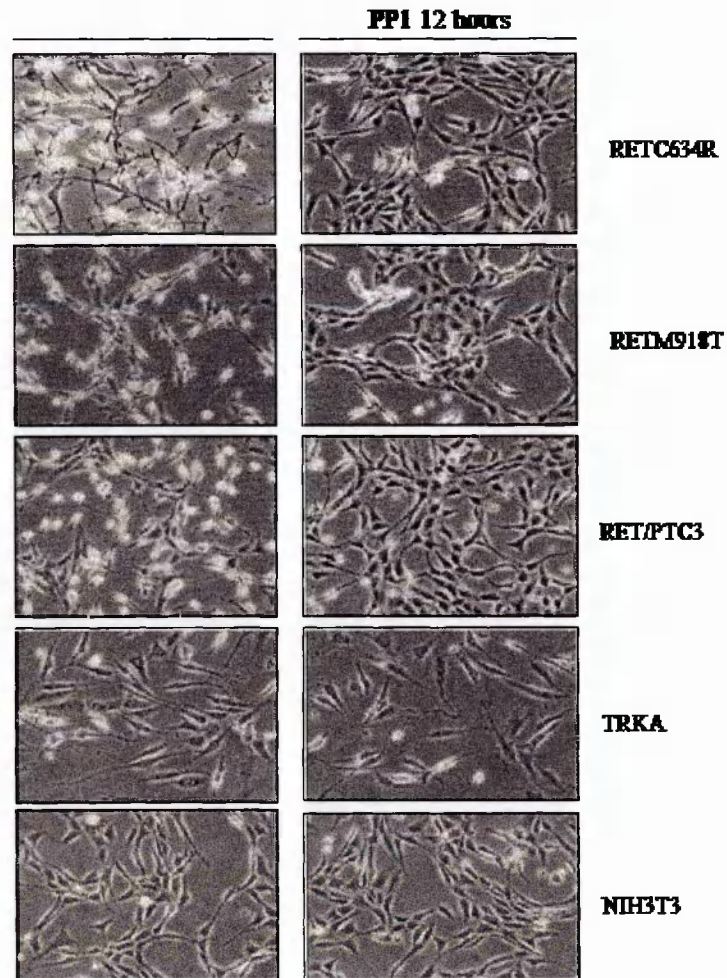


Fig.1

Fig 1: Effects of PP1 on RET^{C634R} , RET^{M918T} and $RET/PTC3$ transformed cells. **(A)** PP1 reverts the transformed morphology of RET^{C634R} , RET^{M918T} and $RET/PTC3$. NIH3T3 fibroblasts stably transfected to express RET^{C634R} , RET^{M918T} and $RET/PTC3$ plated or not in presence of 1 μ M PP1, were analysed 12 hours after plating by phase-contrast microscopy. TRKA-expressing and parental NIH3T3

fibroblasts were used as controls and analysed like the other cell lines.

To further characterise the cytoskeletal rearrangements associated with the morphological changes observed in RET^{C634R}, RET^{M918T} and RET/PTC3 expressing cells upon PP1 treatment, immunofluorescence analysis was performed. Cells were double stained with RET and FITC-labelled phalloidin to examine filamentous actin (F-actin) (Fig. 1B). Cells expressing RET^{C634R}, RET^{M918T} and RET/PTC3 treated with 1 μ M PP1 exhibited a markedly greater amount of actin stress fibers well organised with a pattern similar to that seen for RET negative NIH3T3 cells (Fig. 1B upper panel), when compared to untreated cells. In contrast, parental NIH3T3 cells and NIH3T3 overexpressing TRKA did not show any significant morphological change upon PP1 treatment (Fig. 1B). Moreover, PP1 addition also drastically changed vinculin subcellular localisation in RET^{C634R}, RET^{M918T} and RET/PTC3 transformed fibroblasts (Fig. 1C). In fact, while in untreated cells we found a consistent cytoplasmic pool of vinculin, primarily in the perinuclear region, in PP1 treated cells, a significant fraction of vinculin was seen at the cell periphery in punctuate structures reminiscent of focal adhesion plaques (Fig. 1C). Therefore, the addition of PP1 induced actin stabilisation and

formation of enlarged vinculin-positive focal adhesions, similar to the ones observed in parental NIH3T3 cells, thus reverting the transformed phenotype of RET^{C634R}, RET^{M918T} and RET/PTC3 fibroblasts. PP1 had no effect on vinculin distribution in TRKA expressing cells.

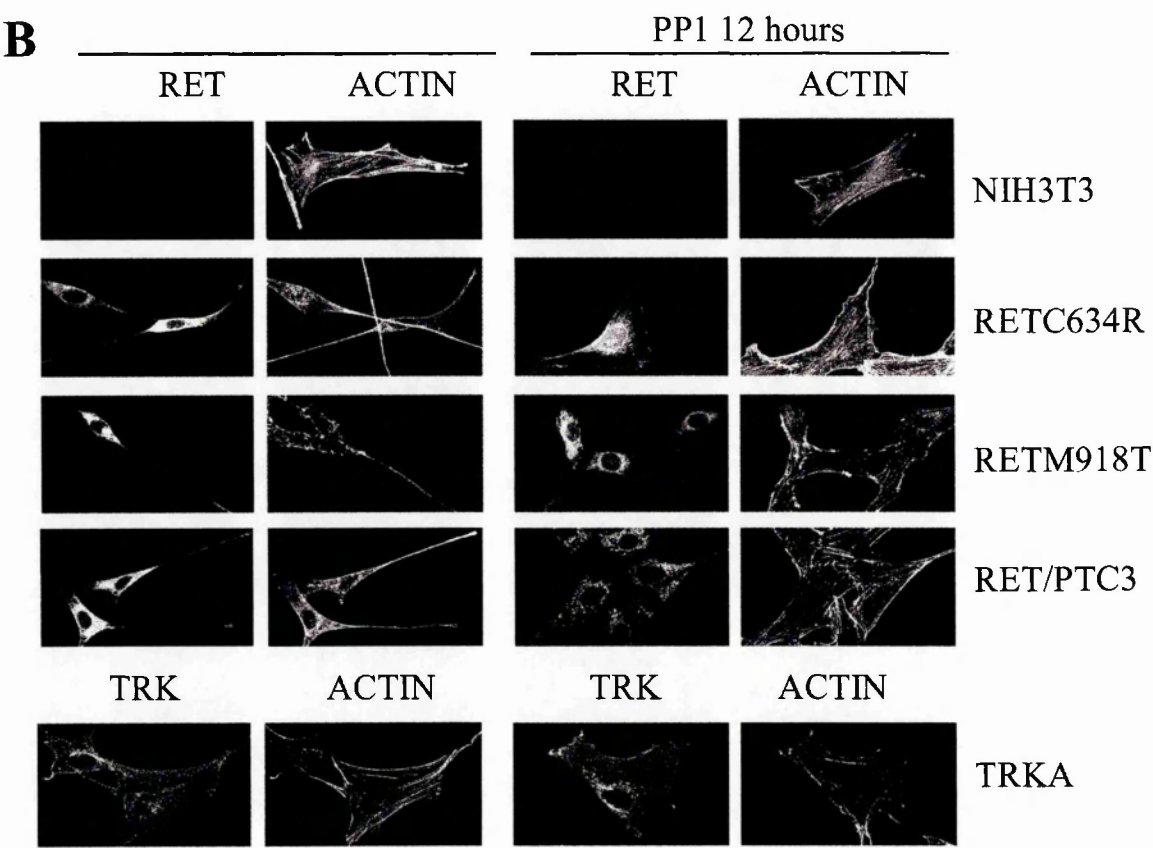


Fig 1

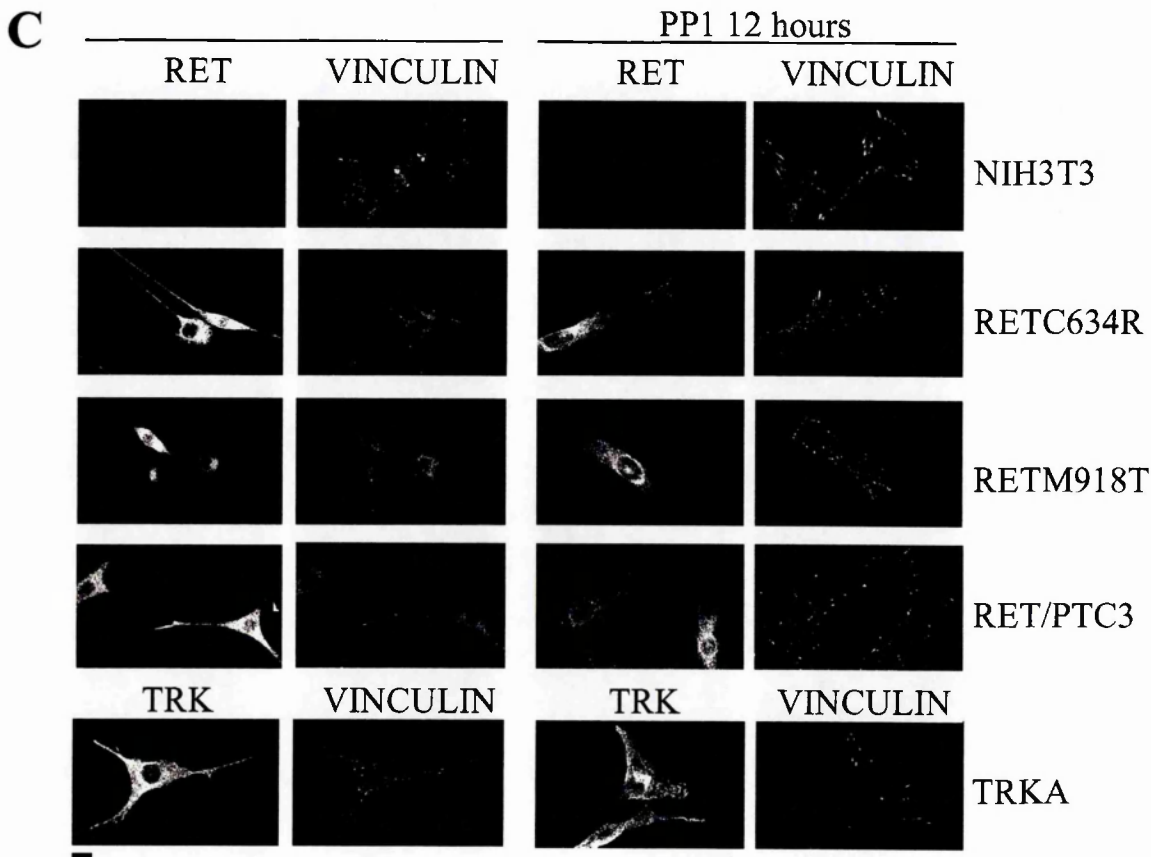


Fig 1

Fig 1: (B) *RET^{C634R}, RET^{M918T} and RET/PTC3 expression in NIH3T3 cells induces changes in actin distribution which are reverted by PP1.* RET and actin distribution in NIH3T3, RET^{C634R}, RET^{M918T}, RET/PTC3 and TRKA expressing cells treated with PP1. Where indicated, transfected cells were plated in the presence of 1 μ M PP1 and after 12

hours from plating, fixed and double-stained with anti-RET antibody and FITC-conjugated phalloidin (actin). **(C)** *PP1 modify vinculin subcellular localisation in RET^{C634R}, RET^{M918T} and RET/PTC3 expressing cells.* To analyse focal contacts, transfected cells were plated in the absence or presence of 1 μ M PP1. After 12 hours from plating, they were fixed and double-stained with anti-RET and vinculin. Images were analysed using confocal microscocopy. Bar: 15 μ m.

An *in vitro* wound repair model (Sato and Rifkin, 1988) was used to assess whether the architectural changes induced by PP1 treatment, could also affect cell motility. Cells including parental NIH3T3 cells, were seeded and cultured until 50% confluence and then wounded, cell culture medium removed and the monolayers incubated. After 12 h from wounding, RET^{C634R} and RET^{M918T} expressing cells demonstrated extensive migration into the denuded area (Fig. 2). In contrast, NIH3T3 untransfected cells showed no significant migratory potential (Fig. 2). When the same experiments were repeated with cells cultured for 12 hours with PP1, we observed that PP1-treatment affected the ability to move, of RET^{C634R} and RET^{M918T} expressing cells whereas the motility of parental NIH3T3 cells was not affected (Fig. 2). This result provided new information on the effects of PP1 on RET transformed cells.

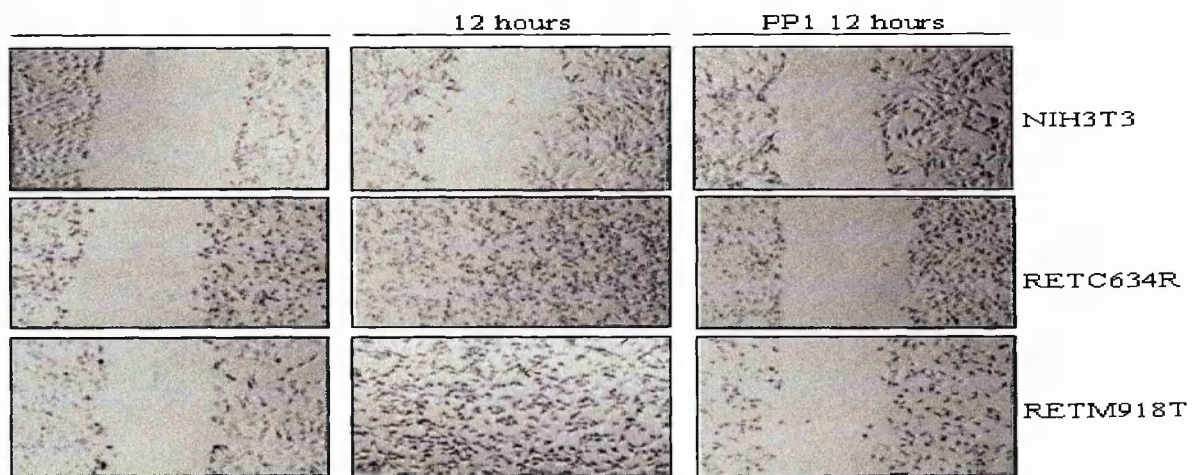


Fig.2

Fig 2: Motility of untransfected NIH3T3 cells, RET^{C634R} and RET^{M918T} expressing cells treated with PP1. Monolayers of untransfected NIH3T3 cells, RET^{C634R} and RET^{M918T} expressing cells were wounded and cultured in presence of serum. Where indicated, 1 μ M PP1 was added for 12 hours. Cells migration in the wound was evaluated at 0 and 12 hours after with a light microscope equipped with phase-contrast optics (original magnification 200x).

4.3 PP1 prevents GDNF mediated scattering of RET expressing SK-N-MC cells.

Ligand-induced activation of RET in SK-N-MC neuroepithelioma cells stably transfected to express RET wild type, generated a "scattering" response, as demonstrated by Van Puijenbroek (Van Puijenbroek et al, 1997). In parental SK-N-MC cells, scattering is also induced by bFGF treatment (Van Puijenbroek et al, 1997). Cell scattering is characterised by several morphological changes such as cellular loosening of epithelial features and cell dissociation with consequent individual movement. In order to determine the effects of PP1 on RET receptor activity, we took advantage of this system and treated SK-N-MC cells stably transfected to express RET wild type and stimulated with GDNF and PP1. The inhibitor was not only able to inhibit GDNF induced scattering but it could also revert the scattering response when given after the morphological changes. If PP1 is given to parental SK-N-MC cells in presence of bFGF, which promotes a scattering response, there are no significant effects. Thus PP1 can specifically inhibit RET mediated scattering but not that induced by bFGF (Fig. 3).

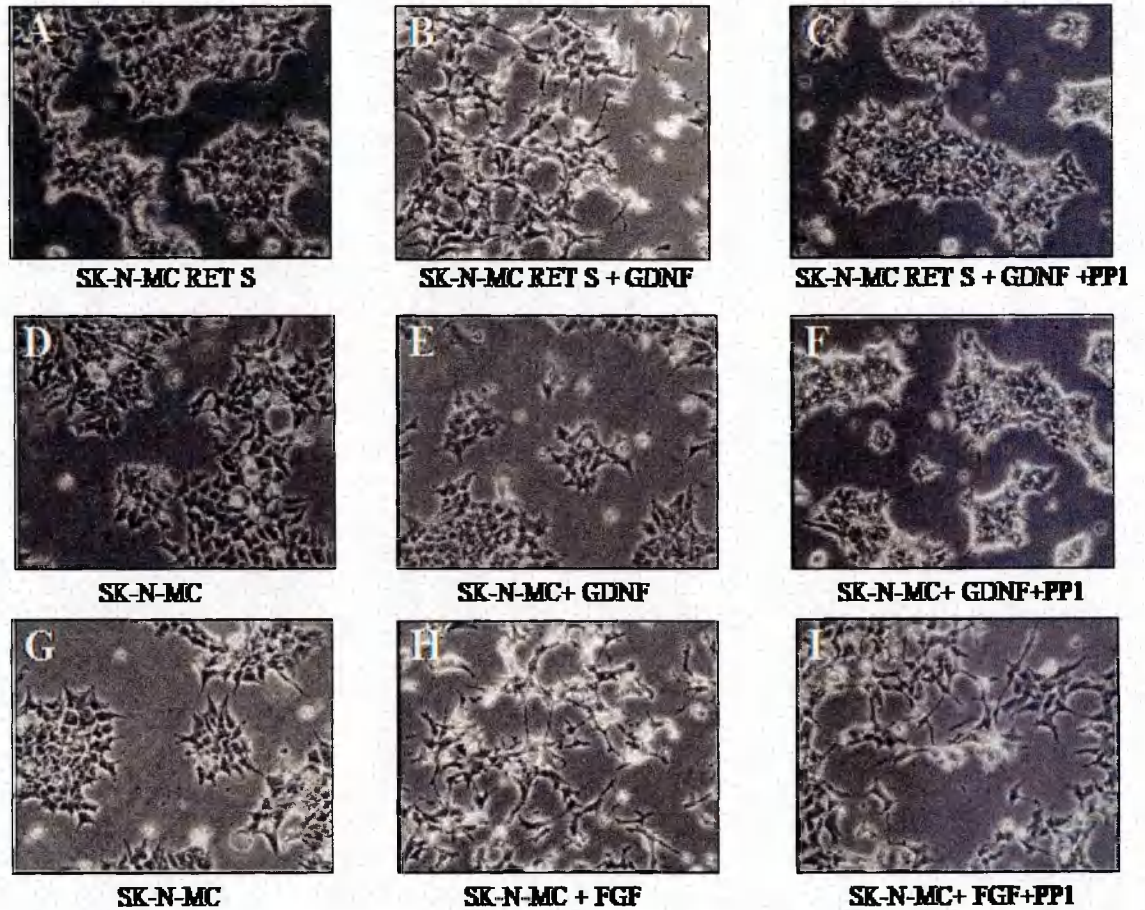


Fig.3

Fig 3: *PP1 prevents GDNF mediated scattering of RET expressing SK-N-MC cells.* SK-N-MC cells, stably transfected to express RET wild type, were serum-starved and stimulated with GDNF or with GDNF together with PP1. As controls, parental SK-N-MC cells were stimulated with bFGF which is known to promote scattering or with bFGF together with PP1.

4.4 Inhibition of RET oncoprotein *in vitro* kinase activity by PP1 and PP2

In order to evaluate RET *in vitro* kinase activity in the presence of the PP1 inhibitor, RET wild type and the RET^{C634R}, RET^{M918T} and RET/PTC3 mutants were transiently transfected into 293T cells and the synthesis of the correctly sized proteins was verified (data not shown). *In vitro* RET kinase activity was dramatically reduced when the assay was performed in the presence of PP1 at a concentration of 1 μ M. As shown (Fig. 4 upper panel), both RET autophosphorylation activity and transphosphorylation of MBP was impaired. As a control, 293T cells were also transiently transfected with a construct coding for the TRKA wild-type protein and enzymatic activity evaluated. In this case, no significant differences were found when the assay was performed in the presence or absence of PP1 inhibitor (Fig. 4 upper panel). These results clearly demonstrated that PP1 was able to strongly reduce the ability of RET to both auto- and transphosphorylate substrates, probably by specifically competing for the ATP binding site as already reported (Carlomagno et al, 2002). To further verify whether PP1 could fit within the RET ATP-binding domain, another inhibitor PP2, which has a chemical structure very similar to that of PP1 (as shown in Figure 4), was used. PP2 mimicked the results obtained with PP1, reducing both RET

autophosphorylation and the phosphorylation of MBP (Fig. 4 lower panel). In contrast, other tyrosine kinase inhibitors such as Genistein or Herbamycin, only partially affected RET enzymatic activity (data not shown and Carlomagno et al, 2002). Taken together, our results strongly suggest that PP1 and PP2 have structures that specifically fit within the ATP binding domain of RET, thus preventing ATP binding.

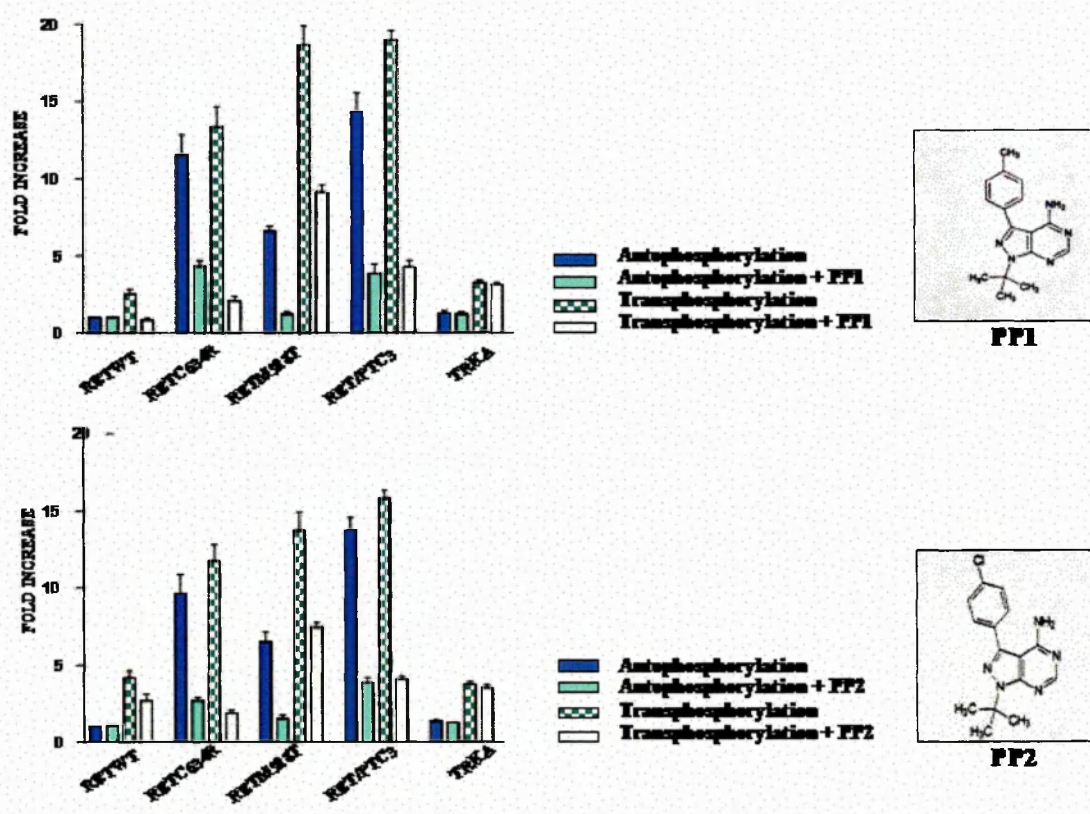


Fig.4

Fig 4: *In vitro* immunocomplex RET kinase assay in presence of PP1. 293 cells were transiently transfected to express RET^{C634R}, RET^{M918T}, PTC3, and TRK. Protein extracts were immunoprecipitated with anti-RET and subjected to kinase assay with MBP as an exogenous

substrate, [γ - 32 P]ATP and PP1 (1 μ M) or [γ - 32 P]ATP. The radio-labelled RET and MBP proteins were resolved by SDS-PAGE and visualised by autoradiography. The intensity of the bands corresponding to autophosphorylated RET and phosphorylated MBP was quantified by PhosphorImager analysis and expressed as the fold increase relative to unstimulated RET wild type. Data represent the mean of three different experiments.

4.5 PP1 treatment of RETMEN2A and RETMEN2B-expressing cells not only affects RET autophosphorylation but induces RET oncoprotein degradation

To assess the effects of PP1 in living cells, RET^{C634R} and RET^{M918T} expressing cells were tested for RET expression and phosphorylation at various times. Parental and TRKA-overexpressing NIH3T3 cells were used as controls. Cells were incubated in the absence (-) or the presence of 1 μ M PP1 for 0, 3, 6, 12, 24, 48 and 72 hours prior to cell lysis. Tyrosine-phosphorylated proteins were detected by western blotting with anti-pTyr antibodies. As expected, PP1 could dramatically reduce the amount of phosphorylated bands in anti-RET immunocomplexes or whole cell lysates (WCL) when visualised on a gel. RET phosphorylation already appeared reduced 3 hours after the addition of PP1 and was observed to remain very low even after 72 hours (Fig. 5A). Surprisingly, when we checked for equivalent protein loading, by re-probing the same blots with anti-RET antibodies, we

discovered that the entire RET protein content of the cell decreased along the time course for both RET^{C634R} and RET^{M918T} (Fig. 5A), but no differences in alpha tubulin content was observed in these cells. Therefore, PP1 was not only able to reduce RET phosphorylation, but unexpectedly, it was also able to cause a progressive loss of RET expression. The effect was specific for RET because PP1 treatment was ineffective on TRKA-over-expressing fibroblasts (Fig. 5B). A progressive loss of RET expression was also observed for RET/PTC3 as shown in Fig. 4B, though the protein level appears clearly reduced after 18 hours. The disappearance of the RET protein was also observed in western blotting experiments when RET^{C634R} and RET^{M918T} cells were treated with PP2 but not when the same cells were cultured in the presence of PP3 (a negative control of PP2 that is able to inhibit the activity of EGFR kinase), or in the presence of other tyrosine kinase inhibitors such as Genistein or Herbamycin (Fig. 5C). This result again indicated that as expected, both PP1 and PP2 exert similar effects on the RET oncoproteins, since they share a common chemical structure. Interestingly, a significant decrease in the amount of RET protein, along with a decrease in its phosphorylation, was detected even when proteins extracted from TT-cells (a human medullary thyroid carcinoma cell line), were immunoprecipitated with anti-RET antibodies and western blot analysis was performed with

anti-RET and anti-pTyr antibodies (Fig. 5D). As expected, in this case, the rate of RET protein degradation was slower than in NIH3T3 cells overexpressing the oncoproteins.

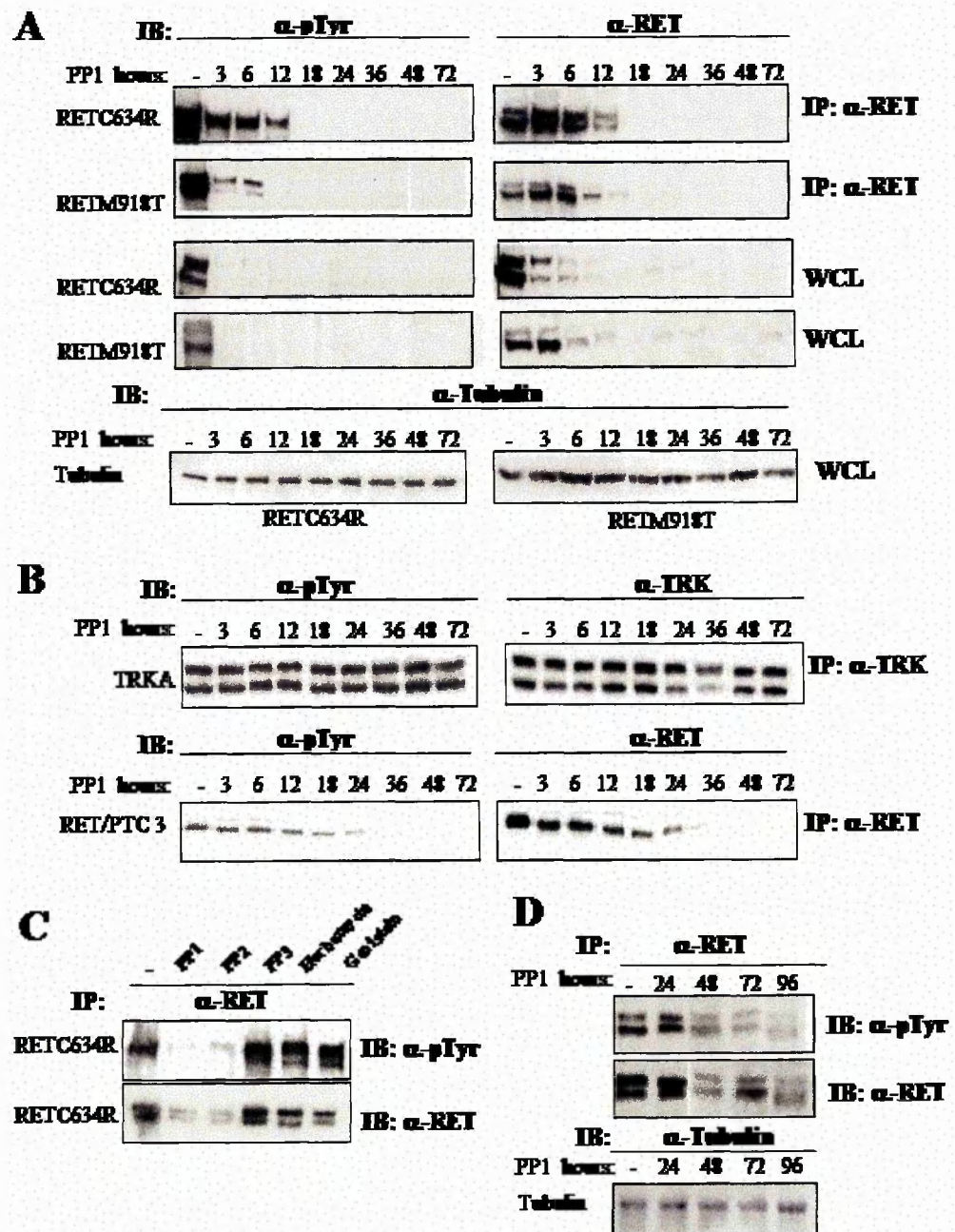


Fig.5

Fig 5: Time course of PP1 treatment. (A) The indicated cell lines were incubated in the absence (-) or presence of 1 μ M of PP1 for the indicated time, prior to cells lysis. Anti-Ret immunocomplexes (Ip) or whole cell lysates (WCL) for RET^{C634R} or RET^{M918T} expressing fibroblasts, were separated on reducing 7% SDS-PAGE and transferred to nitro-cellulose membranes. Upon incubation with anti-pTyr antibodies, tyrosine-phosphorylated proteins were detected by western blotting. After stripping, the amount of protein was verified by re-probing the same blots with anti-RET. Equal protein loading was also confirmed by western blot analysis using anti-tubulin antibody. **(B)** For TRKA overexpressing cells, anti-TRK immunocomplexes (Ip) or whole cell lysates (data not shown) were also separated on reducing 7% SDS-PAGE and transferred to nitro-cellulose membranes. Upon incubation with anti-pTyr antibodies, tyrosine-phosphorylated proteins were detected by western blotting. Anti-TRK antibody was used to verify the amount of proteins on the gel after stripping. For RET/PTC3 anti-RET immunocomplexes (Ip) or whole cell lysates (WCL, not shown) were also analysed for RET phosphorylation and expression. **(C)** The effects of other tyrosine kinase inhibitors were also analysed. Anti-RET immunocomplexes for RET^{C634R} expressing cells treated with PP1 (1 μ M), PP2 (1 μ M), PP3 (1 μ M), Genistein (100 μ M) or Herbamycin (0,1 μ M) for 12 hours were separated on reducing 7% SDS-PAGE and transferred to nitro-cellulose membranes. Upon incubation with anti-pTyr antibodies, tyrosine-phosphorylated proteins were detected by western blotting. After stripping, the amount of protein was verified by re-probing the same blots with anti-RET. **(D)** TT-cells (a human MTC cell line) were immunoprecipitated with anti-RET and western blot analysis with anti-RET and anti-pTyr was performed. Equal protein loading was confirmed by western blotting on the same TT-cell extracts.

Consistent with a progressive loss of RET expression upon PP1 treatment, immunofluorescence analysis of RET^{C634R} expressing cells stained for RET showed that the cellular distribution of the receptor changed during the period of observation. In fact, whereas in untreated cells RET localised both to the cell surface and to intracellular compartments, in PP1 treated cells, staining with specific RET antibodies detected the presence of the mutant receptor mainly in intracellular compartments with RET signal particularly intense in perinuclear regions around the nuclear membrane but largely absent from the cell surface (Fig. 5F). This is particularly evident after 12 hours of PP1 treatment, when RET is still expressed, even though western blot analysis shows a consistent decrease in the amount of protein (Fig. 5A). Consistent with this, staining cells with anti-RET and anti-clathrin antibodies indicated that RET co-localized with clathrin after 20 minutes of PP1 treatment, indicating that PP1 stimulated receptor translocation from the membrane to intracellular clathrin positive structures (Fig 5F bottom panel). This relocalization could be also observed even when immunofluorescence analysis for RET was performed on RET^{M918T} and RET/PTC3 transfected cells treated with PP1 (data not shown). This could indicate that PP1 induces a progressive aggregation of RET molecules into large clusters with a relocalisation to vesicular compartments inside the

cell. During extended PP1 treatments, RET staining tended to disappear progressively, also from the perinuclear region, until no RET staining could be detected (data not shown).

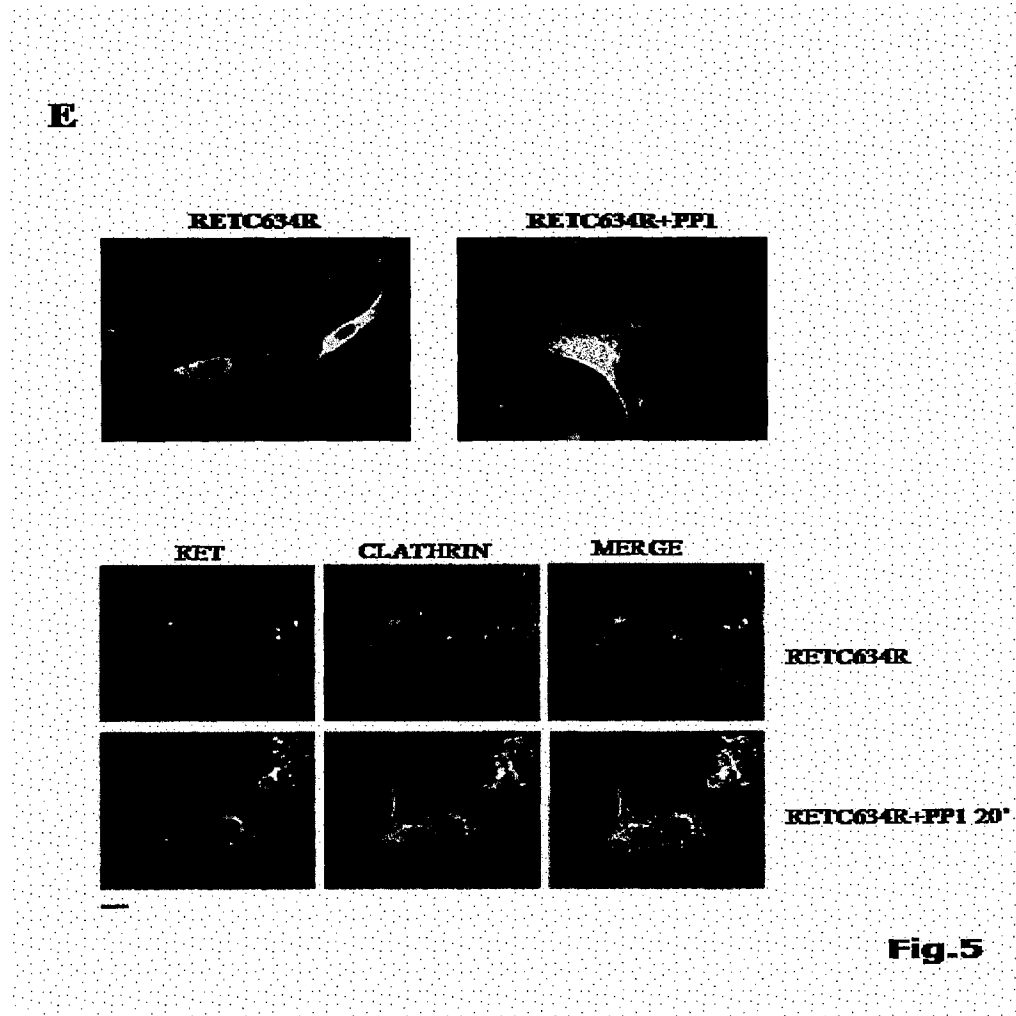


Fig 5: (E) PP1 induces RET redistribution in RET^{C634R} expressing cells. Where indicated, the transfected cells were plated in the presence of 1 μ M PP1 and after 20 minutes from plating, fixed and double-stained with RET (green) and clathrin (red) antibodies. The yellow staining in the merged images indicates co-localization of RET and clathrin after

PP1 treatment (details in the insert). Images were analysed using confocal microscopy. Insert: 4x magnification of normal image; bar: 15 μm .

In order to highlight any significant defect in RET oncoprotein biosynthesis in the presence of PP1, we followed the fate of metabolically labelled mutant RET receptors expressed in NIH3T3 cells. Sub-confluent cultures were labelled with ^{35}S -methionine-cysteine for 20 minutes and subsequently chased for varying times. Cells were previously grown for 12 hours in the presence or absence of PP1 and then metabolic pulse-chase experiments performed as for untreated cells. As already demonstrated by Bongarzone et al. (Bongarzone et al 1999), the amount of RET^{C634R} and RET^{M918T} increased significantly within the first hour of the chase. In PP1 treated cells, the fate of both RET^{C634R} and RET^{M918T} receptors was comparable to that found in untreated cells during the period of observation, indicating that PP1 did not affect protein biosynthesis (data not shown).

4.6 PP1 cytostatic effect on RETC634R and RETM918T expressing cells

We examined the cellular proliferation patterns of RET^{C634R}, RET^{M918T} and RET/PTC3 expressing cells in the presence or absence of the

inhibitor. One micromolar PP1 exerted a remarkable inhibitory effect on the growth of RET^{C634R}, RET^{M918T} and RET/PTC3- expressing cells, as already demonstrated by Carlomagno et al. (Carlomagno et al, 2002). Notably, we observed that this cytostatic effect only started after 48 hours. In fact RET expressing cells resembled parental cells for the first 2 days, but at later times, no increase in the number of live cells in the presence of PP1 could be detected (Fig. 6). This is consistent with the fact that after 48 hours following the addition of PP1 addition, cells had almost completely lost the expression of the RET oncoprotein, suggesting that RET depletion induces growth arrest. No apoptotic effect was observed, as demonstrated by TUNEL analysis, when performed on cells treated with PP1 or PP2 (data not shown). This result was confirmed by western blot analysis using anti-PARP antibodies, performed on whole cell extracts from cells treated with PP1 or PP2 for various times. In fact, no cleavage of PARP, which facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis, was detected (data not shown). The proliferation rate of parental and TRKA overexpressing NIH3T3 cells was only partially slowed in the presence of PP1 (Fig. 5).

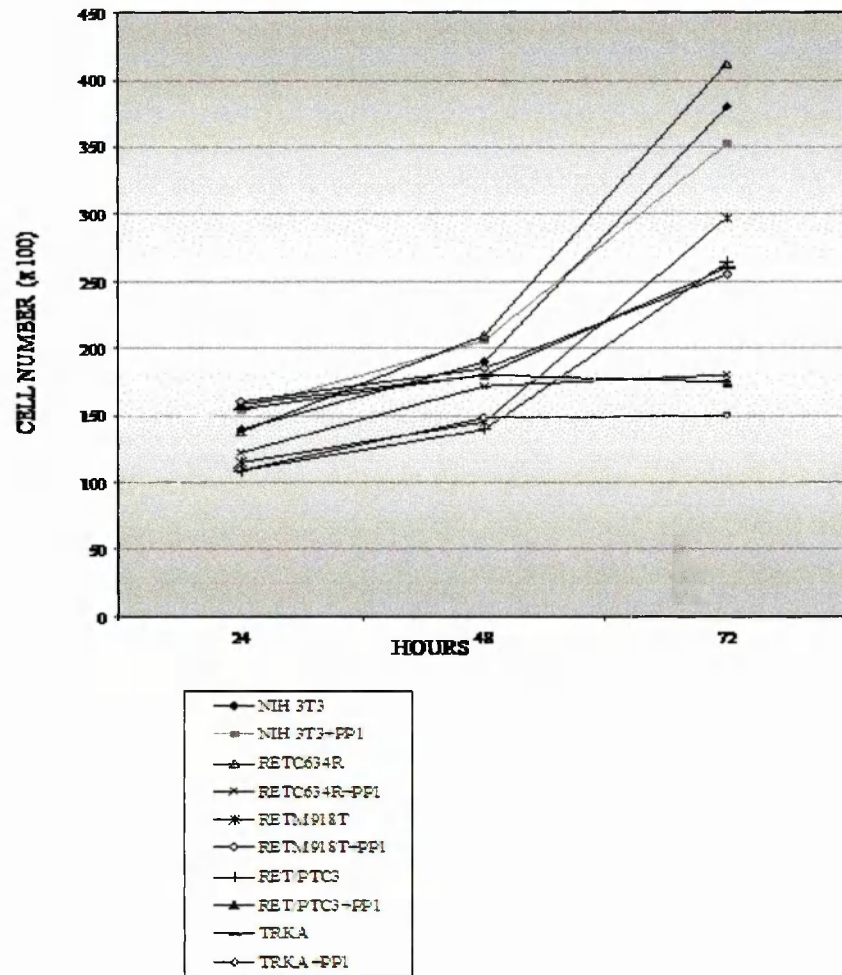


Fig.6

Fig 6: PP1 causes growth inhibition of RET^{C634R} and RET^{M918T} expressing cells. Proliferation of cells grown in the presence of PP1 was assessed. Fifty thousand cells of the indicated cell lines were plated and incubated in medium containing or not 1 μ M PP1. Hour 0 was the treatment starting time. After the indicated period of time, cells were counted and the results plotted on a graph. Data are the mean of three different experiments.

4.7 PP1 treatment induces ubiquitinated RET oncoproteins degradation via proteosomal targeting

We examined whether the PP1 inhibitor could induce RET degradation. Incubation of NIH3T3 fibroblasts expressing RET^{C634R} and RET^{M918T} receptors with both PP1 and the proteasome inhibitor, lactacystin, abolished RET destruction as shown by western blot detection of RET using an anti-RET specific antibody (Fig. 7A). Lactacystin alone did not significantly modify the amount of RET protein. RET degradation was also substantially reduced upon incubating cells with another proteasome inhibitor, MG132 (data not shown). Moreover, RET tyrosine-phosphorylated proteins were detected by western blotting with anti-pTyr antibodies (Fig. 7A). The bands corresponding to RET mutants were not phosphorylated upon treatment with both PP1 and lactacystin, again confirming that PP1 exerts its effect by reducing RET phosphorylation, but that the proteosomal inhibition prevented the destruction of unphosphorylated protein. Taken together, these results strongly indicate that PP1-induced RET degradation is due to proteosomal targeting as the inhibition of the proteasome substantially attenuates the decrease in RET protein levels. Consistent with these observations, chloroquine, a weak base that alkalinises the lysosome, was ineffective in inhibiting RET degradation (Fig. 7A), thus

confirming that PP1 treatment most probably targets RET oncoproteins to proteosomal, rather than lysosomal, degradation. Treatment of the cells with only chloroquine had no effect on RET expression (Fig. 7A). Moreover, neither lactacystin nor chloroquine displayed any detectable effect on TRK expression as detected by western blotting. On the contrary, consistent with previous data (Longva et al, 2002), chloroquine and to a lesser extent also lactaystin, inhibited EGF-mediated degradation of the EGFR in MDA-MB-231 cells, shown by western blotting experiments (Fig. 7A lower panel).

A

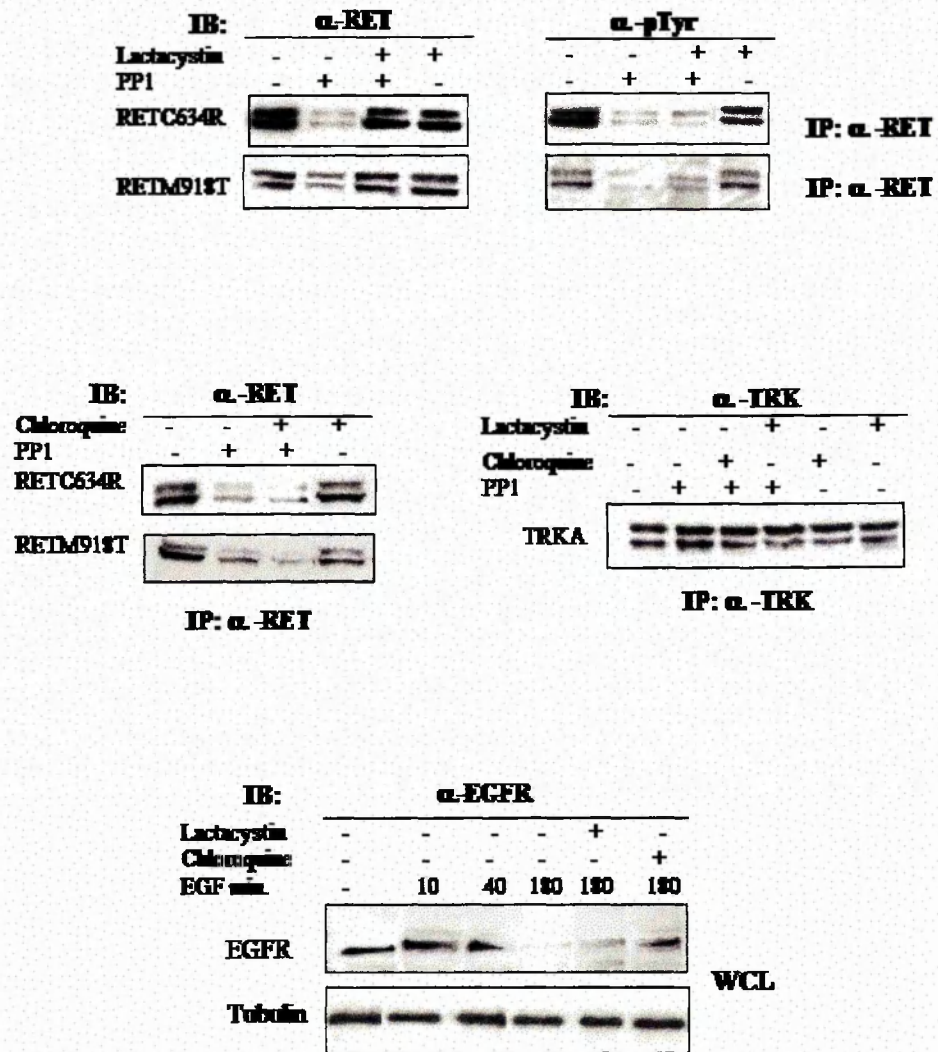


Fig.7

Fig 7: PP1 induces proteosomal rather than lysosomal degradation of ubiquitinated RET oncoproteins which are bound to Cbl. **(A)** NIH3T3 fibroblasts expressing RET^{C634R} and RET^{M918T} receptors were treated with PP1 (1μM for 12 hours), with PP1 (1μM for 12 hours) and

lactacystin (2 μ M for 12 hours) together or with lactacystin (2 μ M for 12 hours) alone. Cell extracts were immunoprecipitated with anti-RET antibodies. Upon incubation with anti-RET antibodies and anti-pTyr antibodies, RET proteins and tyrosine-phosphorylated proteins were detected by western blotting. Proteins were also extracted from RET^{C634R} and RET^{M918T} expressing cells treated with PP1 (1 μ M for 12 hours), with PP1 (1 μ M for 12 hours) and chloroquine (100 μ M for 12 hours) together, or with chloroquine (100 μ M for 12 hours) alone. Western blot analysis of anti-RET immunoprecipitated receptors with anti-RET antibody is shown. To assess the effects of proteosomal and lysosomal inhibitors on EGF-induced degradation, MDA-MB-231 cells were stimulated with EGF (20ng/ml) alone for the indicated time periods or with EGF (20ng/ml) together with lactacystin (2 μ M) for 4 hours or EGF (20ng/ml) and chloroquine (100 μ M) for 3 hours. Whole cell lysates were subjected to western blotting using an anti-EGFR antibody.

Since no previous data exist regarding the physiological down-regulation of RET, interaction with the ubiquitin ligase c-Cbl was also investigated. To assess if c-Cbl could also have a role in RET oncoprotein degradation and whether PP1 treatment could modify RET oncoprotein ability to interact with this ubiquitin ligase, western blot analysis of anti-Cbl immunoprecipitates with anti-RET antibodies from cells treated or untreated with PP1 indicated that RET^{C634R} and RET^{M918T} oncoproteins could associate with c-Cbl (Fig. 7B). Moreover, c-Cbl binds to RET oncoproteins irrespective of PP1 treatment and of

RET phosphorylation status. Western blot analysis of both anti-Cbl immunoprecipitates with anti-RET antibodies (Fig. 7B upper panel) and anti-RET immunoprecipitates with anti-Cbl (Fig. 7B lower panel) in treated or untreated cells demonstrated that RET and c-Cbl can associate *in vivo*, suggesting that c-Cbl interaction with activated RET is an early event not affected by PP1. C-Cbl phosphorylation was also studied in untreated cells and cells treated with PP1. Western blot analysis of c-Cbl immunoprecipitates showed that PP1 does not affect c-Cbl phosphorylation status (Fig. 6B upper panel), but as already demonstrated, dramatically decreased RET phosphorylation and RET protein levels in RET^{C634R} and RET^{M918T} transfected cells (Fig. 7B lower panel). In NIH3T3 untransfected cells, c-Cbl phosphorylation was nearly undetectable and did not change upon PP1 treatment.

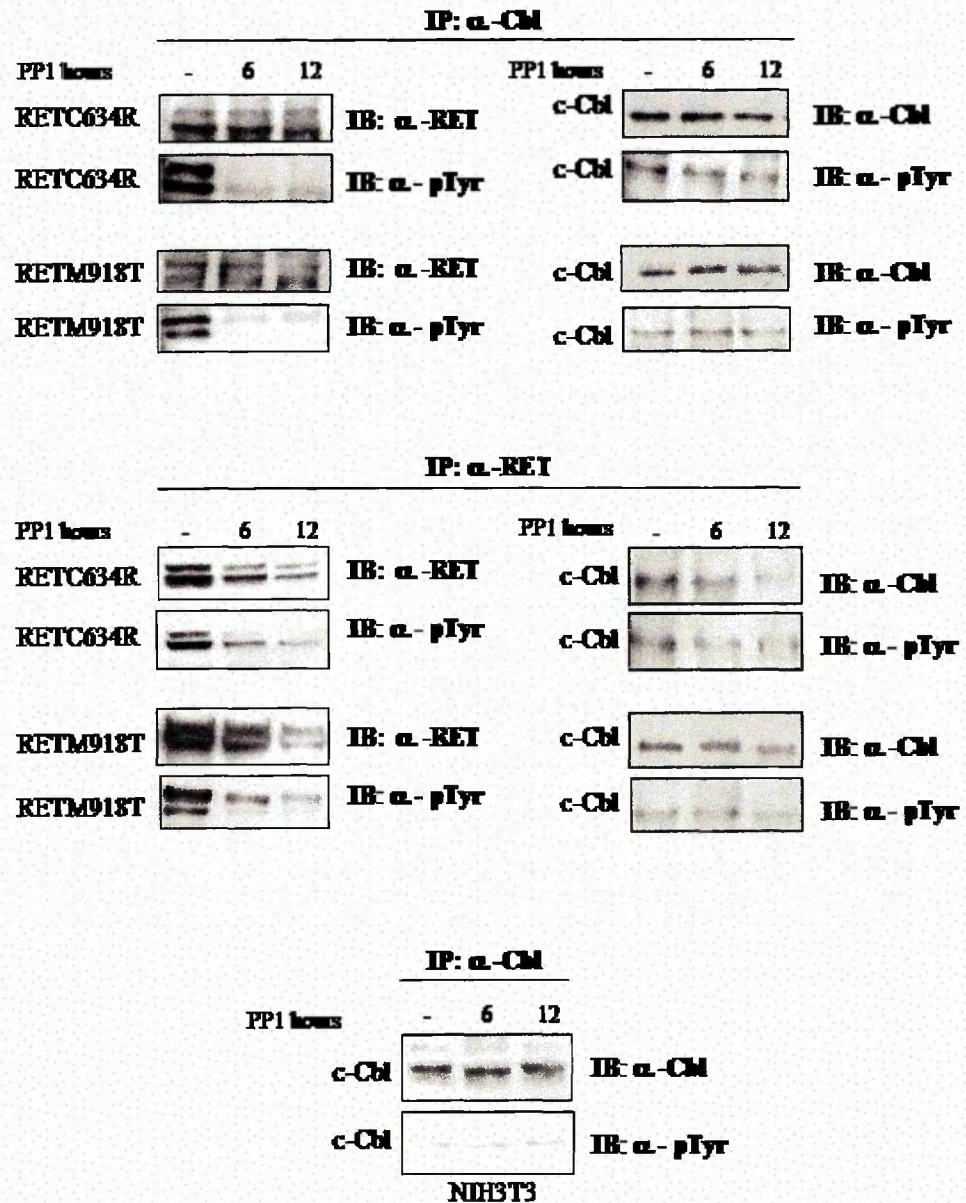
B**Fig.7**

Fig 7: (B) Proteins were extracted from NIH3T3 fibroblasts expressing RET^{C634R} and RET^{M918T} receptors treated with PP1 (1 μ M for 6 and 12 hours as indicated) and c-Cbl immunoprecipitates were subjected to western blot analysis with anti-RET, anti-pTyr and anti-Cbl antibodies to detect RET expression, RET phosphorylation status,

c-Cbl expression and phosphorylation in anti-Cbl immunoprecipitates. To confirm the results, complementary experiments were performed by immunoprecipitating with anti-RET. Anti-RET immunoprecipitates (lower panel) were subjected to western blot analysis with anti-RET, anti-pTyr and anti-Cbl antibodies to detect RET expression, RET phosphorylation, c-Cbl expression and phosphorylation in anti-RET immunoprecipitates. Proteins were also extracted from untransfected NIH3T3 fibroblasts treated with PP1 (1 μ M for 6 and 12 hours as indicated) and c-Cbl immunoprecipitates were subjected to western blot analysis with anti-Cbl and anti-pTyr antibodies to detect c-Cbl expression and phosphorylation in anti-Cbl immunoprecipitates.

Ubiquitination of RET mutants was then studied. Western blot analysis of anti-RET immunoprecipitated receptors, either treated or untreated with PP1 and lactacystin, with a monoclonal antibody against poly-Ubiquitin, indicated that RET^{C634R} and RET^{M918T} oncoproteins were ubiquitinated. Since proteasome inhibition leads to a depletion of free ubiquitin (Patnaik et al, 2000, Schubert et al., 2000, Strack et al., 2000), the signal corresponding to ubiquitinated RET^{C634R} oncoproteins was more intense when cells were cultured in the presence of lactacystin, although ubiquitinated RET could also be seen for RET^{C634R} cells treated with PP1 alone (Fig. 8A). The same results were obtained when analysing the ubiquitination pattern of

RET^{M918T} oncoproteins (data not shown). Additional immunofluorescence studies were performed to confirm that the RET^{C634R} oncoproteins could be ubiquitinated. RET^{C634R} expressing cells were double-stained with anti-RET polyclonal antibodies and monoclonal anti-polyubiquitin antibodies (FK1) to detect ubiquitinated RET mutants. Even in these studies, ubiquitinated RET^{C634R} oncoproteins could be observed in untreated cells (Fig. 8B upper panel and details in Fig. 8B) as well as in PP1 treated cells (Fig. 8B lower panel), thus suggesting that PP1 did not promote ubiquitination. Thus, RET oncoproteins are constitutively ubiquitinated and PP1 treatment does not induce neither affect protein ubiquitination. Taken together, these findings indicate that RET oncoproteins can bind to c-Cbl, can be ubiquitinated and therefore targeted to proteasomal degradation irrespective of PP1 treatment. However, proteasomal degradation is strongly accelerated when cells are treated with PP1.

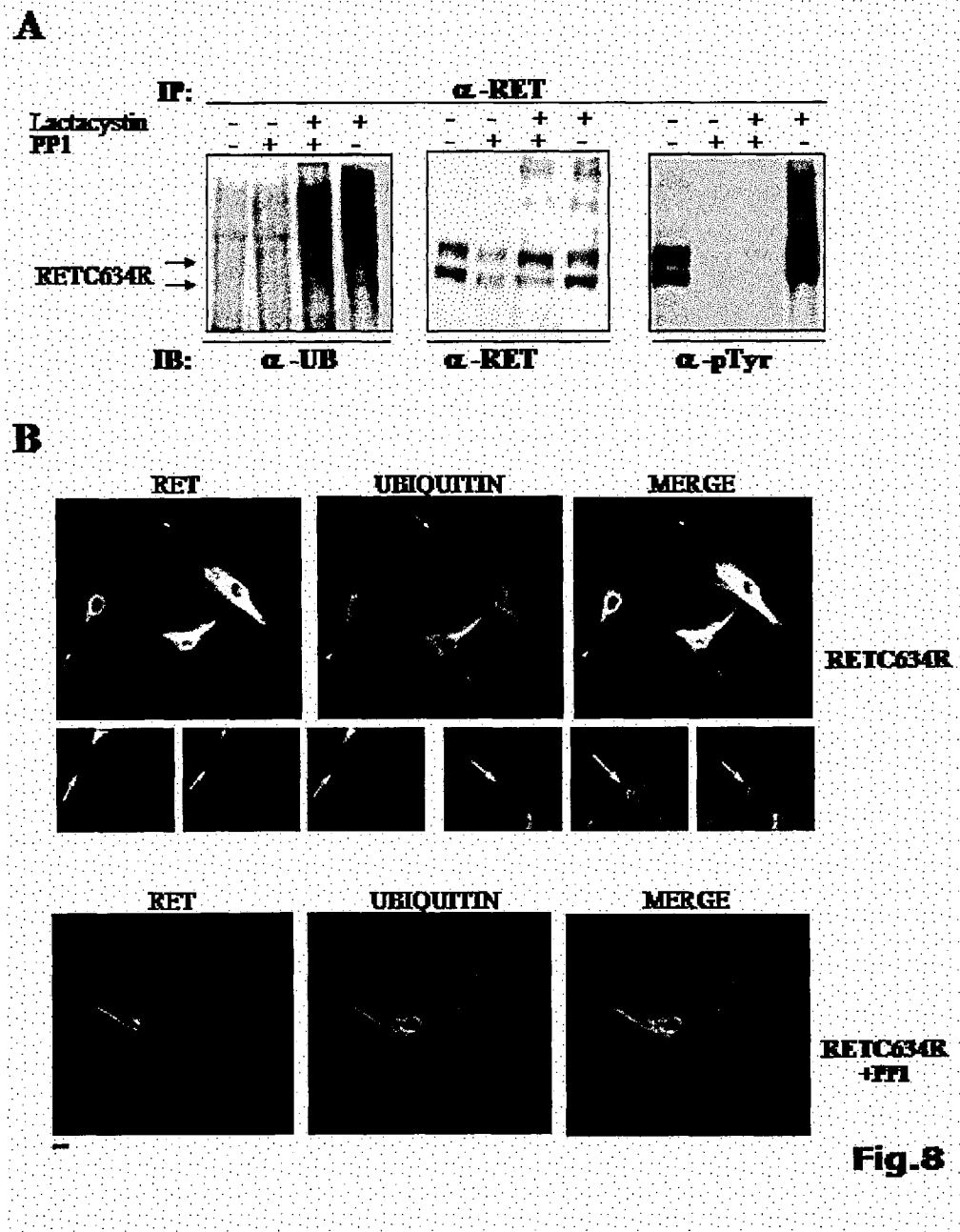


Fig 8: *RET* oncoproteins are constitutively ubiquitinated even in the absence of PP1 (A) NIH3T3 fibroblasts expressing RET^{C634R} receptors were treated with PP1 (1 μ M for 12 hours) or PP1 (1 μ M for 12 hours) and lactacystin (2 μ M for 12 hours) or lactacystin alone (2 μ M for 12 hours). Cell extracts were immunoprecipitated with anti-RET antibodies. Upon incubation with anti-Ubiquitin monoclonal

antibodies, anti-RET antibodies and anti-pTyr antibodies, poly-ubiquitinated RET proteins, RET proteins and tyrosine-phosphorylated proteins were detected by western blotting. (B) NIH3T3 fibroblasts expressing RET^{C634R} receptors, were plated in the absence (upper panels) or in the presence of 1 μ M PP1 (lower panel) and, after 12 hours from plating, fixed and double-stained with anti-RET that was detected by using a rhodamine-conjugated secondary antibody (red) and anti-polyubiquitin monoclonal antibodies that were detected by using a FITC-conjugated secondary antibody (green) and subjected to confocal analysis. Areas of overlap between RET and poly-ubiquitin appear in yellow. The arrows in the upper panel show interesting details reported below at a higher magnification to better illustrate the colocalisation of RET and Ubiquitin. Images were analysed using confocal microscopy. Bar: 15 μ m.

4.8 Discussion

In recent years, several efforts have been made to develop therapeutic strategies targeting oncoproteins. Cancer therapy directed to specific molecular alterations in the signalling pathways of cancer cells has been validated especially for the treatment of advanced breast cancer and gastrointestinal stromal tumors. For ErbB proteins, a group of drugs are already in the advanced stages of clinical testing (Levitzki, 1999; Fry, 2000). These tyrosine kinase inhibitors are very selective, and as a consequence of blocking the

kinase activity most of the down stream signalling pathways are inhibited, leading to growth arrest of tumors dependent on ErbB signalling induced proliferation.

It would be of great interest to identify inhibitors for RET tyrosine kinase in order to develop new therapeutic strategies for RET associated diseases and in particular for medullary thyroid carcinoma, which is the common clinical manifestation of MEN 2 syndromes. MTC responds very poorly to chemotherapy and at present, the only cure involves a total thyroidectomy. Recently, Carlomagno et al. have suggested that PP1 could be used in order to inhibit RET/PTC induced tumorigenesis (Carlomagno et al, 2002).

A key finding made during the course of this present study, which was aimed to discover the effects of PP1 on RET^{C634R} and RET^{M918T} expressing cells, was the unexpected ability of both the two related tyrosine kinase inhibitors, PP1 and PP2, to induce proteosomal destruction of RET oncoproteins. In fact, though PP1 was shown to inhibit RET oncoprotein kinase activity (our data and Carlomagno et al, 2002) and to affect RET/PTC3 dependent MAPK phosphorylation as early as 2 hours after exposure (Carlomagno et al, 2002), analysis of long-term effects of PP1 treatment showed that there was a remarkable decrease in the levels of RET oncoproteins after 12 hours (Fig. 4A) and a clear reversion of the transformed

phenotype was observed (Fig. 1). Moreover, cells expressing RET oncoproteins treated with PP1 proliferated like untreated cells for the first two days after PP1 addition but at later times, a clear cytostatic effect was detectable (Fig. 5) and no RET oncoprotein expression was found (Fig. 4A). Therefore, our observations extend on previous reports stating a linkage between the inhibition of RET phosphorylation and cell growth (Carlomagno et al, 2002), and suggest that drug-induced receptor destruction clearly contributes to the cytostatic effect of tyrosine kinase inhibitors. Moreover, it is tempting to speculate that a stable phenotypical reversion of RET transformed cells and growth arrest might be due to the initial dephosphorylation of RET and its consequent depletion.

No studies have been performed to elucidate the way the RET receptor is physiologically degraded in cells and no data are available on the down regulation of the constitutively activated receptor. For ErbB proteins, two major inducible pathways that control their degradation have been well highlighted: ligand-induced rapid endocytosis of ErbB-1 (Levkowitz et al, 1999), and antibody- and oncogenic mutation-induced destruction of ErbB-2 (Levkowitz et al, 2000), pathways that are both mediated, at least in part, by the c-Cbl ubiquitin ligase. Cbl proteins have a key role in sorting active receptor tyrosine kinases (RTKs) into invaginating pits and function

as ubiquitin protein ligases that direct multi-ubiquitination and downregulation of RTKs. Cbl-mediated ubiquitination accelerates EGFR endocytosis and its delivery to lysosomes for degradation (Levkowitz et al, 1999). Thus, the sorting of RTKs to be internalized, is controlled by two types of reversible modification, phosphorylation and ubiquitination.

The RET mutants associated with MEN2A and MEN2B utilised in this study are constitutively activated and therefore constitutively phosphorylated. Unexpectedly, consistent with this constitutive phosphorylation status of RET oncoproteins, we reported that a high proportion of these oncogenic proteins are ubiquitinated and associate with c-Cbl and ubiquitin. Moreover, treatment of the cells with PP1 does neither promote nor modify this interaction, although the inhibitor abolishes RET oncoprotein phosphorylation but not c-Cbl phosphorylation status. The binding of c-Cbl to RET which had yet to be demonstrated, has now been shown to be an early event for these oncogenic products and that is not affected by PP1-mediated RET dephosphorylation. None of the RET tyrosine residues phosphorylated and/or relevant for RET-mediated mitogenesis seemed to be directly involved in binding c-Cbl, although substitutions of specific tyrosine residues in the intracellular domain of RET have been performed (data not shown). Therefore, the ability of RET to bind c-Cbl can be

separated from its ability to drive mitogenic signalling. Consistent with this, the use of the PP1 inhibitor does not change the ability of c-Cbl to interact with RET, since probably none of the RET tyrosine residues whose phosphorylation is impaired by the inhibitor, are involved in c-Cbl binding. PP1 might then have a role in inhibiting the RET mitogenic signalling cascade but not the pathways that control its degradation. Taken together, our results also give rise to intriguing questions on how the fate of RET oncoproteins is determined since their phosphorylation is constitutive, as is their binding to c-Cbl and their ubiquitination.

Interestingly, Citri et al. (Citri et al, 2002) indicated that the tyrosine kinase inhibitor CI-1033, directed ErbB-2 to a degradative fate mediated by the chaperone destructive system, which is functionally and structurally distinct from the c-Cbl-mediated pathway (Citri et al, 2002). The Cbl-mediated pathway requires kinase activity to induce degradation whereas the chaperone-mediated route is recruited to the kinase domain mainly upon a structural perturbation of the ATP-binding pocket of the oncoprotein. PP1 could act on RET oncoproteins through the activation of similar pathways and the hypothesis that PP1 could target the receptor to destruction by inducing such a structural perturbation, thus recruiting the stress-inducible machinery, is under current study.

In conclusion, we have shown here that the previously reported results on PP1 activity on cytoplasmic RET fusion proteins can be convincingly extended to both membrane-bound RET mutants associated with MEN2A and MEN2B syndromes and to sporadic medullary thyroid carcinomas, emphasizing the potential therapeutic role of this compound. In fact, after PP1 mediated dephosphorylation, RET oncoproteins are rapidly targeted to proteosomal destruction. Interaction with the inhibitor can therefore either block most of the downstream mitogenic signalling, apart from the one driven by c-Cbl so that recycling is prevented and invagination and degradation accelerated, or it can mis-fold the protein structure which is then targeted to stress-induced rapid destruction.

5. CONSTRUCTION AND ANALYSIS OF *retC620R* HOMOZYGOUS MICE

5.1 Introduction

The *c-Ret* proto-oncogene encodes a receptor tyrosine kinase (RTK) that is expressed widely in mammalian embryos and has diverse roles in development and disease (Takahashi et al, 1985, 1988; Taraviras and Pachnis 1999; Baloh et al. 2000; Jhiang 2000). During embryogenesis, the main sites of *c-Ret* expression are the excretory and nervous systems (Pachnis et al. 1993; Tsuzuki et al. 1995). In the nervous system, *c-Ret* is expressed in the progenitors of the enteric nervous system (ENS), in enteric, autonomic, and sensory neurons of the peripheral nervous system (PNS), and in motor and catecholaminergic neurons of the central nervous system (CNS). The majority of enteric neurons and glia are derived from a subset of neural crest (NC) cells that emigrate from the neural tube at the level of somites 1-7. On invading the foregut mesenchyme, enteric NC (ENC) cells migrate in a rostrocaudal direction and colonize the wall of the gastrointestinal tract (Kapur et al. 1992; Durbec et al. 1996). Mutations of *c-RET* in humans lead to absence of enteric ganglia from the distal colon and congenital megacolon

(Hirschsprung's disease, HSCR; Parisi and Kapur 2000), whereas *Ret.k⁻/Ret.k⁻* mice lack all enteric ganglia posterior to the stomach (intestinal aganglionosis; Schuchardt et al. 1994; Durbec et al. 1996). Although some of the cellular processes controlled by RET (such as survival, migration, and differentiation of ENC) have been identified, the mechanisms that lead to the localized absence of enteric ganglia in HSCR patients remain unclear. Gain-of-function mutations of *c-Ret* have also been described in humans and reproduced in mice by targeted mutagenesis. These mutations result in the constitutive activation of RET and are associated with multiple endocrine neoplasia (MEN) types 2A and 2B and familial medullary thyroid carcinoma (FMTC), inherited cancer syndromes characterized by tumors of neuroendocrine origin (Jhiang 2000; Smith-Hicks et al. 2000). Although MEN 2 mutant forms of the RET protein efficiently transform fibroblasts (our results), their biological effects in mice have remained elusive and until now, no model mimicking the co-occurrence of HSCR and MEN2A was available.

I have targeted the mouse *RET* locus by homologous recombination to generate a strain of mice that carries the C620R RET mutation in order to study the role of the RET^{C620R} mutation *in vivo* and hopefully have the first mouse model which could be studied in terms of tumor development and developmental

disorders such as HSCR disease. Heterozygous mice are viable. In contrast, homozygous mice expressing the RET^{C620R} mutation die early after birth and lack kidneys. This is in line with previous findings showing that mice homozygous for a targeted mutation of *c-Ret* (*Ret.k^{-/-}*) have severe hypodysplasia or aplasia of the kidneys (Schuchardt et al. 1994, 1996; Durbec et al. 1996; Srinivas et al. 1999) and die within 24 hours after birth. The excretory system of knockout RET mice exhibited a wide spectrum of abnormalities ranging in severity from bilateral or unilateral renal agenesis, blind-ending ureters with no renal tissue, to small and dysplastic kidney rudiments. The mammalian kidney is generated by reciprocal inductive interactions between the ureteric bud and the undifferentiated metanephric mesenchyme. Whereas the mesenchyme is important for the growth and branching of the UB, which gives rise to the renal collecting system, the tips of the UB branches induce the surrounding mesenchymal cells to condense into epithelial vesicles, which differentiate into the various segments of the nephrons (Saxen and Sariola, 1987). In mice, *c-Ret* mRNA is first detected in the nephric duct of the pronephros and mesonephros at E8.5-E10.5, with the highest levels at the caudal end of the duct, from which the ureteric bud later evaginates. At E11.5, when the UB has branched within the metanephric

mesenchyme, c-Ret is expressed throughout the ureteric bud and its function is necessary for evagination, growth, and branching of this structure (Pachnis et al. 1993; Schuchardt et al. 1994, 1996; Sainio et al. 1997; Ehrenfels et al. 1999; Schedl and Hastie 2000).

5.2 Construction of the Transgene

In order to target the mouse RET locus by homologous recombination in embryonic stem (ES) cells, we first isolated a ret genomic clone by screening a mouse 129 lambda bacteriophage DNA library (129/Sv) using RET cDNA containing exon 10 as a probe. The targeting vector, was constructed using a 1.8kb 5' BamHI-BamHI fragment and a contiguous 4kb BamHI-SacI 3' fragment containing exons 10 and 11 in pBluescript SK (Stratagene) followed by site-directed mutagenesis using the QuickChange kit (Stratagene) and PAGE-purified mutagenic primers. The pGKneoTK selection cassette (a gift from T.H. Rabbits), flanked by loxP sites to allow its removal by the Cre recombinase was inserted into the BglII site immediately downstream of exon 11. The diphtheria toxin A chain gene contained within the pKOselectDT plasmid (Stratagene; Greenfield et al. 1983, GenBank Acc. # K01722) was inserted into the 5' RsrII site, such that it was outside the 5' region of homology

(Fig.1). The targeting construct was linearised with NotI and electroporated into CCB ES cells (a gift from M.J. Evans) derived from the 129S7 strain. ES cells were plated on mitomycin C-treated primary embryo feeder cells grown on gelatin and obtained from a Rosa26 x MF1 cross (and therefore G418-resistant) followed by selection in G418.

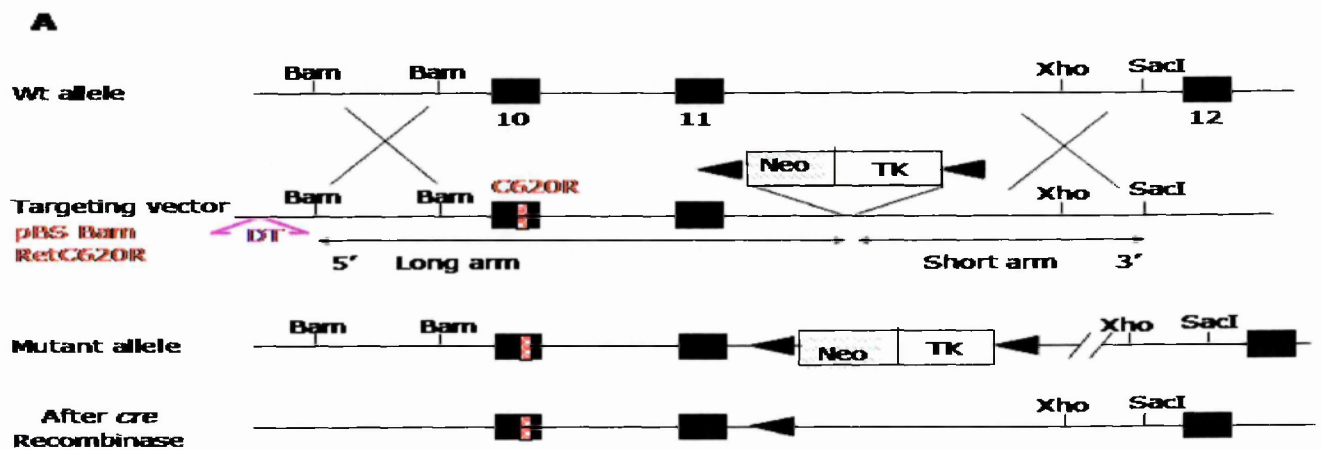


Fig 1

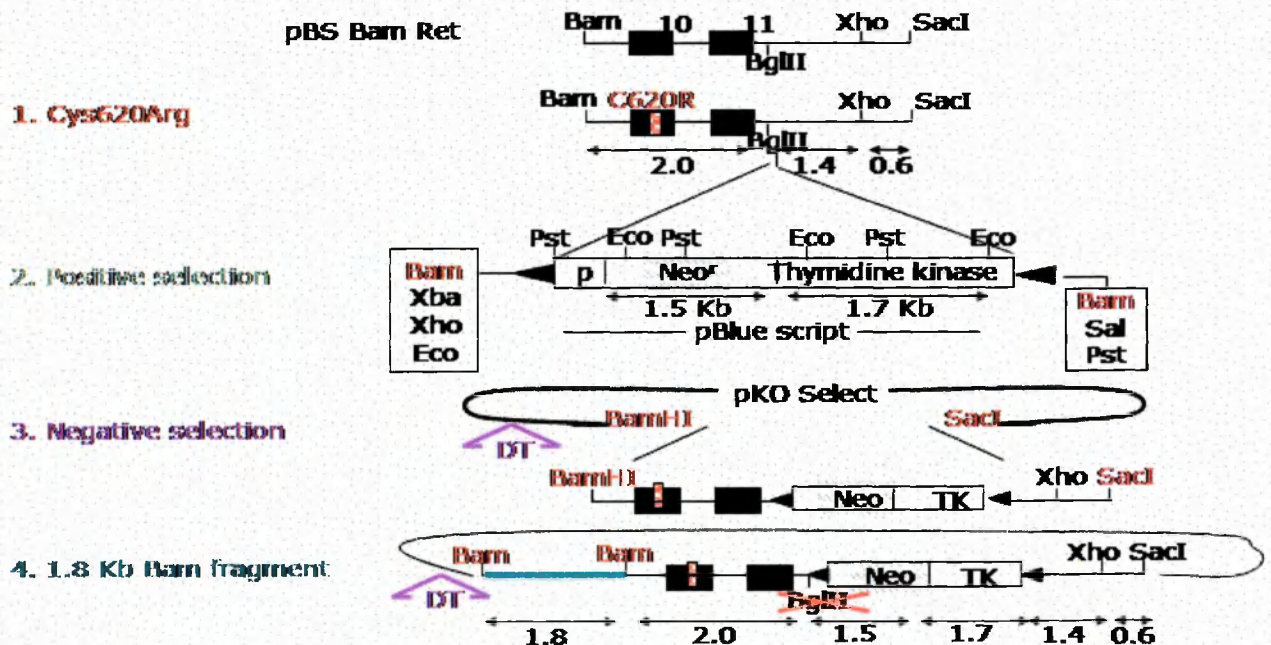
B**Fig 1**

Fig. 1 The targeting the murine *RET*^{C620R} locus: **A.** "Touch and go" homologous recombination for *Ret* "knock-in". The targeting vector was constructed using a 1.8kb 5' BamHI-BamHI fragment and contiguous 4kb BamHI-SacI 3' fragment containing exons 10 and 11 followed by site-directed mutagenesis. The pGKneoTK selection cassette flanked by loxP sites to allow its removal by the Cre recombinase, was inserted into the BglII site immediately downstream of exon 11. The diphtheria toxin A chain gene contained within the pKOselectDT plasmid was inserted into the 5' RsrII site such that it was outside the 5' region of homology. The targeting construct was linearised with NotI. **B.** Details of the construction of the targeting vector *pBS Bam RetC620R*.

Of 197 G418-resistant clones, 8 (4%) had the targeted insertion according to the applied PCR screening method (P1, 5'-aagctctctagtcgaggaat-3' and P2, 5'-ggcctctgaaggactgaa-3') (Fig 2A). Southern blotting confirmed that 4 of these had the targeted allele of the expected size (Fig 2B). DNA sequencing confirmed that 2 clones (1%) had the C620R RET mutation (not shown). The floxed selection cassette was excised in vitro in the RET C620R-neoTK/+ ES using pCre-Pac (a gift from D.J. Winton). Of 161 clones, 11 had undergone cre-mediated excision of the selection cassette as shown by PCR analysis performed on the DNA of the ES cell clones (Fig 2C). Only 2 clones were confirmed positive when sequencing analysis was performed (data not shown). One targeted *ret* C620R/+ ES cell clone was expanded and microinjected into C57B1/6J blastocysts at the Gene Targeting Facility, Babraham Institute, Cambridge, to obtain chimaeric mice. Of 146 live-born pups, this clone gave rise to 18 highly chimaeric animals in which the percentage chimaerism was exceptionally good (1x100%, 7x90%, 8x80%, 1x70%, 1x50%). The chimaera with the greatest ES cell contribution was chosen and used to transmit the mutation through the germline to 4 of 8 pups. The genotypes of these animals were confirmed using a two-primer assay (P1, 5'-tgccgacattgttgagggaac-3' and P2, 5'-

cctggctgttcctctggct-3'). A 120 base-pair bp amplification product is generated from the wild-type allele which is not cleaved after digestion with the restriction enzyme BSTUI that instead, gives a 98bp fragment for the allele bearing the C620R substitution. Animals were bred to C57BL/6J mice to generation N3 before intercrossing and therefore segregate C57BL/NJ and 129S/ alleles in the ratio of 6:1, respectively. The official nomenclature for this mouse line is, C57BL/6JN3TgH(ret620)Ac620, which has been abbreviated to B6N3Ac620 and hereafter referred to as retC620R.

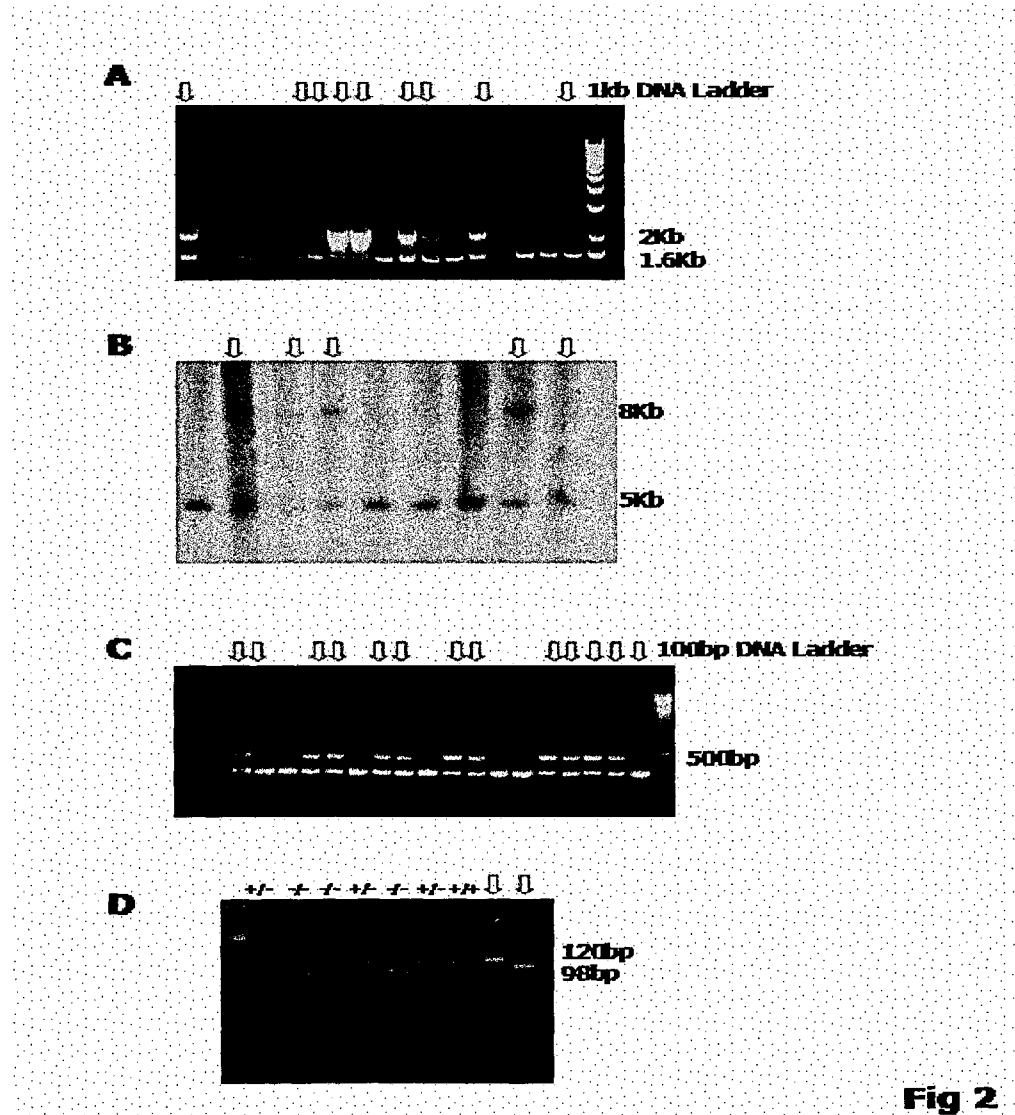


Fig 2

Fig. 2 *Detection of homologous recombination events in the ES clones.* **A.** DNA was extracted from the 197 G418-resistant ES cell clones, 8 (4%) had the targeted insertion according to the PCR screening method (P1, 5'-aagctctctagtcgaggaat-3' and P2, 5'-ggcctctgaaggactgaa-3') which amplifies a 2kb fragment in the 8 targeted clones (blue arrows) and a 1.5 Kb fragment in the wild type (red arrow) and in the non targeted clones. **B.** Southern blotting confirmed that 4 of the above 8 clones had the targeted

allele of the expected size of 8kb (blue arrows) compared to the wild type size of 5kb (red arrow). The DNA was digested with BamHI and hybridized to a probe obtained by amplifying a fragment containing ret exon 11 and therefore present in both the targeted and non-targeted clones (P1, 5'-acagggggaggtggtacagt-3' and P2, 5'-atgccgtatccaccatctgt-3'). **C.** PCR-based analysis of the clones after the electroporation of the Cre-recombinase. Of 161 clones, 11 had undergone cre-mediated excision of the selection cassette and displayed the correct expected size (blue arrows) after DNA amplification through the region of the insertion of the cassette as a loxP site remains and gives a band of approximately 500bp compared to the wild type (red arrow) and the non targeted that are shorter (450bp) (P1, 5'-cagggcttcccaatcagtt-3' and P2, 5'-ccgagtacatgtgtgcctgt-3'). **D.** PCR-based analysis of representative tail biopsies from a litter of pups of one heterozygous breeding pair. A 120 base-pair bp amplification product (P1, 5'-tgccgacattggtggagggaac-3' and P2, 5'-cctggctgttcctctggct-3') is generated from the wild-type allele which is not cleaved after digestion with the restriction enzyme BSTUI that instead, gives a 98bp fragment for the allele bearing the C620R substitution. The products of the amplification and digestion of control wild type DNA (red arrow) and DNA from the targeted retC620R vector (blue arrow) are loaded on the gel as controls. The resulting genotype of each pup is indicated. -/- Wild type; +/- heterozygous; ++ homozygous mutant.

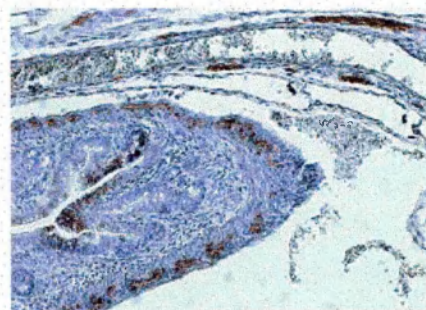
5.3 *retC620R* homozygous mice die early postnatally

All heterozygous *retC620R* mice appeared healthy and grew and reproduced normally. The genotype distribution of the new-born mice obtained from *retC620R*^{+/-} intercrosses was as follows: wild type 24%; heterozygous 51% and homozygous 25%, indicating that there was no significant increase in embryonic lethality of homozygous *retC620R* mice. At birth, homozygous mutants were the same size as their wild-type and heterozygous littermates, responded to tail pinch, and nursed normally. However, all *retC620R* homozygous mice, died within 24 hours of birth. The mice were all analyzed for the inheritance of the *retC620R* transgene through the extraction of genomic DNA from tail samples collected from all the littermates.

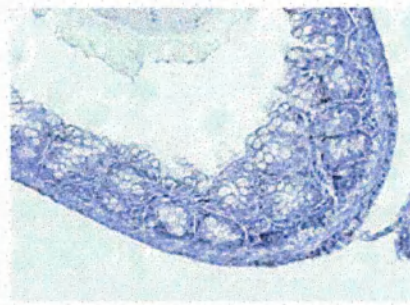
5.4 Enteric Nervous System Deficits and Kidney agenesis in *retC620R* homozygous mice

Dissection of newborn homozygous *retC620R* mice revealed that many of them had milk in the esophagus (which was not observed in wild-type animals) and little milk beyond the proximal small bowel, suggesting defects in gastrointestinal peristalsis. Given the absence of enteric neurons beyond the

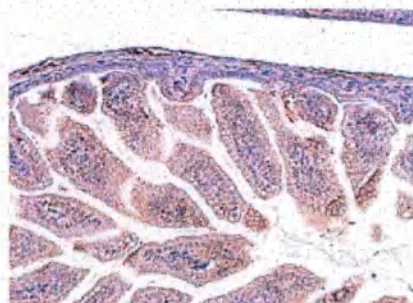
stomach in Ret-, GDNF- and GFRalpha1- deficient animals, it seemed likely that these observations similarly reflected defects in the enteric nervous system of our mice. Microscopic analysis and immunohistochemistry, with antibodies specific for neuron-specific enolase (NSE) demonstrated that enteric ganglion cells were absent in the esophagus, stomach, in the small or large bowel of retC620R animals (Fig.3) but they were present in both the wild type and heterozygous mice.



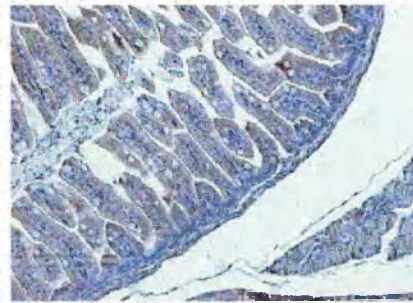
Colon wt NSE 10X



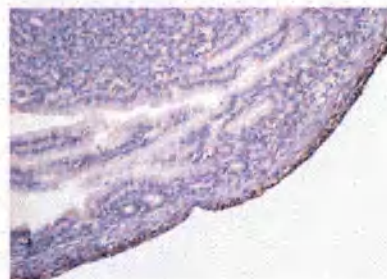
Colon RetC620R NSE 10X



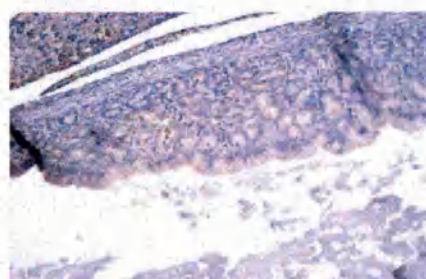
Small intestine wt NSE 10X



Small intestine RetC620R NSE 10X



Stomach wt NSE 10X



Stomach RetC620R NSE 10X

Fig. 3 Histological Analysis of the Enteric Nervous System in RetC620R mice: Neuron-specific enolase (NSE) staining demonstrates readily identified ganglion cells in the wall of the intestine and stomach just in wild type animals whereas in RetC620R mice no staining is present.

Moreover, it was found that the homozygous newborn animals had agenesis of the kidneys as well as empty bladders (Fig. 4). All of them lacked both kidneys, consistent with what was observed for mice lacking Ret, GDNF or GFRalpha1. Renal abnormalities in heterozygous retC620R mice was searched for, but all of the mice examined had two kidneys, undistinguishable from those in the wild-type animals. This is similar to what was reported for both Ret^{+/-} mice (Schuchardt et al., 1994) and GFRalpha1^{+/-} mice (Enomoto et al., 1998), but in sharp contrast to what was observed in GDNF^{+/-} mice where up to 30% of heterozygotes displayed kidney abnormalities (Sanchez et al., 1996).

The adrenal glands and components of the urogenital system including the testes, vas deferens, ovaries, oviducts and uteri, appeared macroscopically normal.

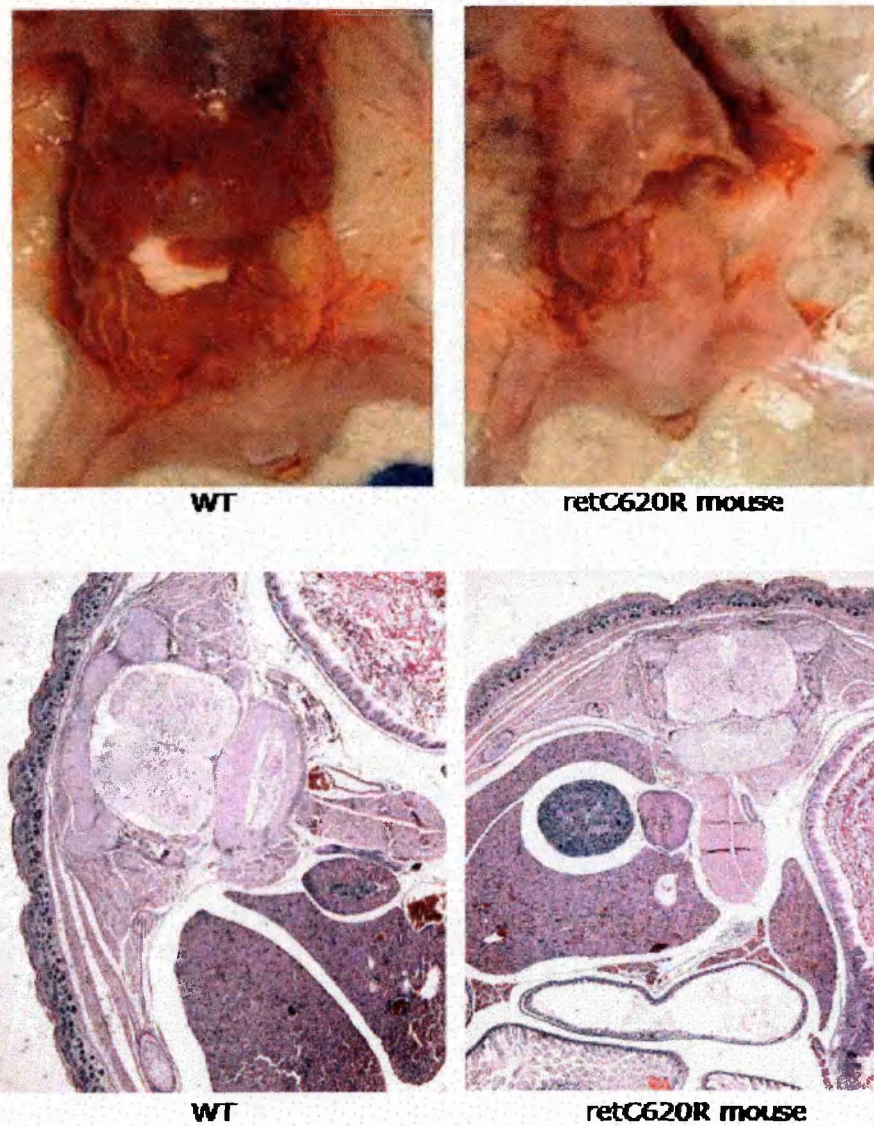


Fig 4

Fig. 4 *Kidney agenesis in retC620rR mouse.* No renal tissue could be observed in retC620R homozygous mice even when all the organs hiding the kidneys were removed for a clear observation. The wild type and heterozygous mice had normal urogenital systems. Hematoxylin/Eosin sections are shown in the lower panel.

5.5 Discussion

Previous studies have established the absolute requirement of GDNF, RET and GFRalpha1 in the development of the enteric nervous system and kidneys. I have shown here that mice homozygous for *retC620R* lack kidneys and have defects in the enteric nervous system, as do knock-out mice for GDNF, RET and GFRalpha1. In fact, these events lead to the death of all the *retC620R* homozygous animals as is the case in RET, GDNF and GFRalpha1 knockouts. These results indicate that the RET mutant we introduced completely impaired the development of the affected organs and thus the signals triggered by the RET, GDNF and GFRalpha1 complex are impaired.

I have already shown in the previous chapters of this thesis, through the biochemical analysis of the transfected RET mutants in NIH3T3 cells, that the RETC620R protein hardly translocate to the cell membrane. Most of it was retained in the endoplasmic reticulum where the mutated protein could not interact with either GDNF or GFRalpha1 and thus trigger the signals necessary for the development of the kidneys. It was also shown that the RETC620R mutants did not respond to GDNF, since SK-N-MC cells did not scatter when ligand is added.

Although very preliminary, these results strengthen the idea, which was already proposed during the course of this study, suggesting that the reason why the RETC620R mutation is concomitantly associated with both the MEN2A syndrome and HSCR disease relies on the fact that for an efficient development of both the kidney and the enteric nervous system, RET needs to be present at the cell surface signaling through the interaction with GDNF and its coreceptor. On the other hand, as well demonstrated biochemically in this thesis, RET can signal even from the endoplasmic reticulum where the RETC620R mutant is retained and bind to the mitogenic components of the cascade essential to drive mitogenesis. In humans in fact, this mutations never occurs in homozygosis since probably no individuals would survive due to the severe defects that the lack of at least one copy of RET wild type could cause.

One of the oldest animals we have now seems to present an enlargement of the thyroid, probably indicating the presence of a tumor but we have no data on tumor formation in these mice as they are just 10 months old and we have yet to sacrifice any of them. These mice with could be crossed to other strains to study whether the same mutation induces

different phenotypes when inserted in diverse genetic backgrounds. A veterinary unit will analyze the histology of the homozygous mice which died just after birth, since we are also interested in obtaining some more insights into the nervous system of these mice and look for other abnormalities similar to those found in RET, GDNF and GRFalpha1 knockouts.

6. CONCLUSIONS

The overall aim of this work was to investigate the expression of different RET mutants in order to highlight their biological role in diverse cellular contexts. In particular, this thesis focuses on gain of function Cys mutations that are responsible for medullary thyroid carcinoma by causing the covalent dimerization of RET, leading to ligand-independent activation of its tyrosine kinase. In this context, the association of Cys⁶⁰⁹ and Cys⁶²⁰ activating mutations with HSCR is still an unresolved paradox. In the attempt to clarify why an activating mutation can also give rise to the absence of enteric neurons, I have produced several lines of evidence that suggest that the mitogenic signaling responsible for growth and development of medullary thyroid carcinoma does not require either the presence of the RET protein at the cell surface or the formation of the RET/ligand/coreceptor complex. On the other hand, this complex is strictly necessary for the correct development of the enteric nervous system and the excretory system. Therefore, the presence of a functional RET at the cell surface is absolutely critical in order to mediate the effects of GDNF during renal and enteric nervous system development.

For the first time, mice bearing a single substitution at RET codon C620R were generated and represented a very useful

tool in our study. When these mice were bred to homozygosity they died early post natally and completely lacked an excretory system. There is evidence that suggests that similarities exist between these mice and mice lacking either GDNF or RET or GRFR α 1. All three mutant animals show absolute losses of enteric ganglion cells in the distal gut (small intestine and colon), whereas some enteric neurons remain in the proximal gut (stomach and esophagus), characteristics that have been also found in our mice.

The generated mutant animal could also represent a very interesting model in which to study Medullary Thyroid Carcinoma development and growth. One of the oldest mice we have (10 months old), presents as expected, a macroscopically detectable thyroid enlargement that is probably a tumor formation. Based on the results of a recent paper by Cranston A (Cranston AN and Ponder BA, 2003), I too would like breed these animals and introduce the mutation into different genetic backgrounds to study any eventual differences in the rate of tumor growth in order to identify possible genes that predispose for the disease. It was demonstrated that the occurrence of tumours in RET transgenic mice depends on the genetic background of the host (Cranston AN and Ponder BA, 2003). Still in collaboration with Dr. Cranston, I am trying to create a transgenic animal which expresses the RET^{C634R} proto-

oncogene with exactly the same procedure applied for the generation of the RET^{C620R} mice. ES cells clones obtained after electroporation with the plasmid bearing the mutation need to be tested for the presence of RET^{C634R} and to be used for morula aggregation in order to obtain the transgenic animal. Future studies will then focus on the differences between the retC620R and the retC634R mice.

These animals are possibly a good source of tumor cell lines and good models in which to study how the inhibitor we studied and others drugs work *in vivo*. During the course of this study, a new and interesting potential treatment strategy, using PP1 inhibitor was proposed. This inhibitor, originally described as a Src family kinase inhibitor, rapidly affects RETMEN2A and RETMEN2B oncoprotein phosphorylation and targets them to the degradative pathway. PP1 treatment would represent a promising new strategy to selectively target RET oncogenic products for destruction and could be used to develop new therapeutic techniques for Medullary Thyroid Cancer therapy whose tumors respond very poorly to chemotherapeutic agents. The obtained results are also relevant since no studies have been performed to elucidate the way the RET receptor becomes physiologically degraded in cells and no data are available on the mechanisms controlling the down regulation of constitutively activated receptors. They also give rise to

intriguing questions on how the fate of RET oncoproteins is determined as their phosphorylation is constitutive as is their binding to c-Cbl and their ubiquitination.

Further studies will focus on the different pattern of protein expression of cells bearing RET proto-oncogenes treated or not with the PP1 inhibitor. These experiments will be performed by two-dimensional electrophoresis approach followed by analysis of the expressed proteins using Mass Spectrometry. Preliminary results have already indicated consistent differences in the "phosphoproteome" of the cell lines when treated or not with PP1.

The results obtained, when testing the PP1 inhibitor on various RET mutants, prompted us to try to re-classify the different RET mutation according to their responsiveness to PP1: one of the hypothesis we favour in explaining the activity of the inhibitor is that the degradative signals recruited to the kinase are mainly due to structural perturbation of the ATP-binding pocket of the oncoprotein, where PP1 might enter and physically interact with RET. RET mutants bearing substitutions in the ATP-binding domain behave differently when treated with PP1 with respect to the exact location of the substitution. This is the subject of a new article that was submitted for publication together with Dr. Cranston A. in collaboration with Dr. Taylor S. and her crystallography group.

Conclusions

In conclusion, this study gives rise to a number of interesting issues that need to be further investigated, utilizing also the animal model designed for the study of MEN2A and MEN2A/HSCR.

Acknowledgements

The last lines of this text should be words of gratitude to all those who helped to make this thesis possible. First of all, I have to thank Tati Bongarzone and Prof. Bruce Ponder for being patient supervisors and for supporting this work with ideas and criticism. I am very thankful to Carla Perego who did great work in implementing the ideas presented in this thesis and whose support made it possible to extend my research into the area of fluorescence confocal imaging. Piera Mondellini helped me with technical problems, Baharia Mograbi provided me with some clones of the SKNMC cells. I also want to thank Arlette Franchi who taught me everything I know on ES cells and for the joined work on the RETC620R transgenic mice. For providing me with an environment and support for my work I am very grateful to Marco Pierotti. Together with Aaron Cranston and Baharia Mograbi I had numerous discussions, more or less related to this thesis. I would like to thank Manuela Campiglio for the gift of the MDA-MB-231 cell line and for the EGFR antibody and Simona Polo for the gift of the clathrin antibody and for critical review of my manuscripts. Sara Bellucco did with the histological analysis of the retC620R mice. Over the years I have been fortunate to enjoy stimulating discussions with a number of colleagues whose advice was invaluable to me. I am deeply grateful for their support. Last, but not least, I am indebted

Acknowledgments

to my family for their patience and I need to thank Debora, Ritz and Dario for standing by me in good and bad times, thank you all!

The research described in my PhD thesis has been generously supported by the TELETHON Foundation (grant n°E0993) and by the Italian Association for Cancer Research (AIRC).

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